CD4+ T-cell subsets in rheumatoid arthritis

The exact pathogenesis of rheumatoid arthritis (RA) remains uncertain, however, autoimmune processes appear critical. In the past decades, several models implicated T cells at different levels; however, recent genetic advances have clearly indicated a role for T cells, maybe somehow limited to autoantibody positive disease. Processes involved in aging seem to occur early in RA and deviation from normal physiological pathways such as repertoire diversification, signaling, differentiation, polarisation or regulation also characterized T cells from RA patients. Despite such evidence, T-cell targeted therapies did not appear to be particularly successful with the exception of costimulation blockade, the reasons for this failure remaining unclear. Importantly, T-cell subsets demonstrated interesting biomarker features that remain to be investigated in relation with early diagnostic, prognostic and prediction of treatment response.

KEYWORDS: naive T cell ▪ regulatory T cells ▪ rheumatoid arthritis ▪ Th1/Th2/Th17 subsets

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Evidence for roles of T cells in rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints that results in disability and premature mortality [1–3]. Its exact pathogenesis remains uncertain. However, autoimmune processes appear critical as evidenced by major histocompatibility complex linkage [4,5], the presence of circulating autoantibodies (rheumatoid factor, anticollagen antibodies and more recently anti-citrullinated peptide antibodies [ACPA]) and the infiltration of the synovial tissue with lymphocytes often arranged in aggregates or forming ectopic germinal center-like structures [6,7]. In the 1980s, this evidence led to a model in which T cells orchestrate the local inflammatory response with an arthrogenic agent (virus, bacterial or autoantigen) stimulating T cells to expand locally, activating the local environment (fibroblasts and endothelial cells) to recruit other immune cells (B cells and macrophages) [8]. The idea that synovitis was an antibody-mediated process was abandoned in the 1990s in favor of a multiple cell type-mediated model involving antigen-presenting cells, B and T cells with a major role for macrophages. Notably at this time evidence emerged for the presence of macrophage-derived products in the joint, while it was more difficult to detect T cell-derived cytokines [9,10]. A few years later, and increasingly, accumulating evidence points to a major role for synovial fibroblasts [11,12].

The original 'T-centric paradigm' however, also presented a number of difficulties. The model centers on activated T cells, but in RA, infiltrating T cells appear predominantly inactive [13]. As a result of autoantigen stimulation, synovial T cells should be clonal. Although T cells specific for autoantigens have been isolated from the joint [14–16], polyclonality is more often observed [17,18]. T cell-derived cytokines should be produced at the disease site and although some T cell cytokines are present they are not in abundance and are actually produced by non-T cells [9,19]. Combined with the failure to identify a common antigen (either native antigen, neo-antigen released during inflammation or an infectious agent) and the lack of IL-2 production [9], this evidence led to the concept that T cells in RA have only a 'passive or irrelevant role' [13]. It remains difficult to resolve these competing schools of thought and the role of T cells may be more crucial in the initial phases of RA whereas the evidence for a T-cell primacy in established, chronic synovitis remains more controversial, suggesting that disease duration may indeed be a major factor. The characterization of particular states of T-cell differentiation such as Treg and, more recently, Th17 cells may help to elucidate the roles of T cells in RA. In this review, we will discuss work on the general features of T cells in RA and in more detail, CD4+ T-cell subsets.

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General features of T cells in RA

Genetic susceptibility

Heritability in RA has been estimated at approximately 60%, with similar results in ACPA-positive and ACPA-negative disease [20]. The association between susceptibility to develop RA and the inheritance of several human leukocyte antigens (HLA) class II alleles, that are involved in the presentation of antigens to T cells, is well established [21]. Indeed, the *HLA-DRB1* genes have shown their importance in RA susceptibility, notably for the DR1 and DR4 alleles carrying the now well-known 'shared epitope' amino acid motifs that share common biochemical properties and was shown to facilitate RA development and progression [22]. The contribution of the *HLA* genes to the overall genetic risk in RA has been estimated to range from 30 to 50% [23,24].

Recent genome-wide association studies, suggested a list of approximately 30 loci associated with RA in addition to gender [25–27]. However, the individual contribution of any one of these genes is very small and combined they contribute to less than 5% of the variance. Several loci are implicated in T-cell responses (PTPN22, CD2 and STAT4) and cross-talk between T cells and other immune cells (CD40, CD28, CTLA4 chemokines and receptors) [28,29], strongly suggesting a genetic influence involving T cells independently and in addition to the *HLA* genes in RA susceptibility. A gene–gene interaction between two polymorphisms of the TNF receptor (TNF-R) superfamily, TNFRSF14 and TNFRSF6B has also been demonstrated [30]. Interestingly the TNFRSF6B protein is involved in the modulation of T-cell activation and differentiation and its ligand is overexpressed in RA synovium. Finally, a role for IL-12 in RA susceptibility has more recently been proposed. IL-12 is an inducer of IFN-γ production and is involved in driving T cells towards Th1 polarization. There was no direct association between IL-12 gene polymorphisms and RA [31–33] however, associations were reported with polymorphisms in the signal transducer and activator of the transcription 4 (*STAT4*) gene, which encodes a transcription factor involved in the signaling pathways of IL-12 and IL-23 [34].

T-cell aging & RA

Aging of T cells limits their ability to proliferate through telomere shortening. It has been a major limitation in immune reconstitution following high-dose chemotherapy in cancer [35], an issue that is now being addressed by supplementation of IL-7 post-therapy to increase T-cell development. An unexpected consequence of T-cell depleting therapies in RA was prolonged peripheral blood lymphopenia, mostly affecting the $CD4^+$ subset, for up to 7 years following both depleting mAb therapy [36,37] or autologous stem cell transplantation [38–40]. Although the implications of these findings remain uncertain, it was remarkable that these patients did not suffer increased mortality or infectious/malignant complications despite prolonged $CD4^+$ T-cell lymphopenia [37]. One possible explanation may be that peripheral blood analysis was misleading and central lymphoid tissues were relatively unaffected. Alternatively, chronic exposure to inflammation may have impaired the ability of the RA immune system to reconstitute. Accelerated T-cell aging combined with telomeric shortening has however been associated with autoimmune responses, notably in RA [41,42], and RA $CD4^+$ T cells specifically exhibit premature aging, as evidenced by a shortening of their average telomere length [43]. Aging of the immune system is also associated with a progressive loss of responsiveness, resulting in increased mortality and morbidity. Such age-associated changes have been attributed to both T and B cells, and is often associated with chronic low-grade inflammation (termed 'inflamm-aging') [44]. Both naive and memory T cells in RA displayed poor homeostatic proliferation in response to lymphopenia and this was the main factor limiting reconstitution. Using measurement of T-cell receptor excision circles [45] and analysis of T-cell subset differentiation (naive and memory cells [46]) we confirmed that the cause of prolonged lymphopenia in RA was not related to thymic deficiency. In RA indeed, the thymus has an intact reserve although it exhibits a very slow and diminished response to lymphopenia [47]. We associated these deficiencies with an RA-specific lack of IL-7 response to lymphopenia [47]. To date, the mechanism by which this response is lost remains unclear however we have hypothesized that exposure to TNF-α may have a negative effect on production of circulating IL-7 as shown for stromal cells in the bone marrow [47].

T-cell repertoire distortion in RA

Significant distortions of the T-cell receptor (TCR) repertoire were reported in RA [17,48]. Usage of TCR β-chain sequences in $CD4^+$ T cells in healthy individuals shows high diversity with all sequences used. In RA patients however, certain β-chains showed tenfold higher

usage whereas others were absent, suggesting marked contraction [48]. Such dominant clonotypes, preferentially utilizing V-β 3, 14 and 17, were identified in the synovial fluid CD4+ T-cell repertoire suggesting further local clonal expansion [17]. Similar usage of V-β was reported for CD8+ T cells [49,50]. However, this phenomenon was not restricted to the memory compartment but also observed in the naive CD4+ T-cell repertoire [48] suggesting abnormalities in the generation of the repertoire rather than shaping of the repertoire by antigen-driven proliferations. It was proposed that these alterations were imposed by the HLA-DRB1 allele itself, its expression resulting in the preferential selection of certain V-β segments and also J-β elements in healthy controls [51,52]. This imposes a particular rigidity on the TCR molecule and contributes to shaping of both naive and total CD4+ T-cell repertoire whereas responses to antigen encountered in the environment mostly add shaping to the memory T-cell repertoire. Clone frequencies also varied independently of each other, suggesting active stimulation by several antigens.

Abnormal signaling

RA patients have an increased vulnerability to infections and malignancies, particularly malignancies of the immune system [1,53]. Although some of this immune dysfunction may be attributable to immunosuppressive therapy and nonspecific effects of chronic disease, there is also evidence implicating intrinsic disease factors. For example, T-cell Ca^{2+} responses from RA patients were globally reduced with a large proportion of cells not responding at all [54]. Antigen stimulation-mediated proliferation was also reduced. However, the surface expression of TCR (CD3) was not altered suggesting abnormalities downstream of the cell surface signal acquisition, specifically the tyrosine phosphorylation of the TCR-ζ chain and phosphorylation cascade between LAT and Zap70 [55,56]. Furthermore, RA synovial T cells, despite evidence of previous activation (memory phenotype), showed hyporesponsiveness to mitogen stimulation compared with blood and hardly produced any cytokines [57]. This later defect was associated with a deficit in redox-regulating enzyme (glutathione) [56,58]. Chronic exposure of T cells to TNF-α also inhibits signaling through the TCR [59]. Anti-TNF therapy in RA patients however, reverses some of these *in vivo* and *in vitro* defects, most notably the TCR [59] and calcium signaling [37,60]. Abnormal RAP1 signaling during TCR stimulation was also reported in RA synovial T cells [61,62] and restoration of RAP1 signaling in the collagen-induced arthritis model suggested potential therapeutic benefit [63].

A mouse model with spontaneous development of arthritis by 6–7 months of age has brought further evidence that dysregulated T-cell signaling may give rise to arthritis [64,65]. In this model, the gp130 signaling chain shared by several cytokine receptors (including IL-6, IL-11, LIF, oncostatin M and others) was mutated ($gp130^{F759/F759}$) in order to enhance signaling, bypassing a negative feedback loop by which phosphorylation of a tyrosine in position 759 would attenuate gp130 signaling. These mice are born normal but develop a phenotype associated with activated T cells, sustained activation of JAK-1 and STAT-3 (even in the absence of IL-6), the presence of autoantibodies, as well as the perturbation of thymic selection allowing auto-reactive T cells to escape selection. CD4+ T-cell proliferation was necessary for the development of arthritis [66] and memory T cells were also predominant and hyper-reactive to lower levels of stimulation [64]. The development of the disease was shown to be dependent on CD4+ T cells but also on cytokine (but not necessarily IL-6) signaling through gp130 in nonhematopoietic stromal cells [66]. Conditional inhibition of STAT3 activation in stromal cells in these mice resulted in specific downregulation of IL-7 production but had no effect when performed in lymphoid cells. Anti-IL-7 antibodies were sufficient to abrogate both T-cell proliferation and disease development. In another mouse model, the SKG-$ZAP70^{W163C}$ mice, also develop spontaneous arthritis with synovitis, autoantibodies and bone and cartilage erosion [67]. In this model however, T cells were hyporesponsive and thymic selection was perturbed allowing specific selection of arthritogenic T cells. With a lower threshold of signal being required to develop Th1 response, these T cells appeared to be activated by cytokines expressed locally in the synovial environment rather than by antigens, although, thymic alteration of the development of regulatory mechanisms (Treg, naturally occurring Treg) was also invoked to explain how hyporesponsive T cells could be responsible for the development of disease.

T-cell subsets in RA

Naive & memory subsets

One of the main characteristics of the immune system is its ability to develop memory over time. With aging, existing naive T cells are preserved through slow turnover and long lifespan [58,68],

however the thymus slowly loses its ability to produce new naive cells (notably illustrated in [46]). The breadth of the naive T-cell repertoire is therefore slowly reduced with consequences for older individuals in both their ability to respond to new antigen stimulation and vaccination, and a predisposition to cancer [69]. Conversely, cumulative exposure to foreign pathogens and environmental antigens promotes the accumulation of memory T cells with age [70]. Isoforms of the tyrosine phosphatase CD45, along with expression of a number of other cell surface markers are frequently used to distinguish 'naive' from 'memory' T cells. Classically, CD45RA expression declines following activation of naive CD45RA$^+$ cells, with a concomitant rise in the expression of CD45RO. In RA, this classic differentiation model is perturbed and double-positive CD45RA$^+$/CD45RO$^+$ are present [46]. L-selectin (CD62L) is another classic naive T-cell marker (CD45RA$^+$CD62L$^+$) with homing for lymph nodes. In RA, CD62L is lost on a subset of CD45RA$^+$ cells [46], however, these cells remain naive with respect to antigen stimulation [46] but express chemokine receptors for trafficking to sites of inflammation [71]. We hypothesized that this is the result of cytokine activation of naive T cells enabling the need for an antigen to be bypassed [68,72].

CD27, a member of the TNF-R family, is expressed on naive T cells, and is gradually switched off in effector/memory cells [73]. CD27 expression distinguishes two subsets of CD4$^+$ CD45RO$^+$ memory T cells: effector cells CD27-, displaying a high antigen recall response and a resting population CD27$^+$ or central memory population, lacking antigen recall response, and requiring costimulation for re-activation [74]. This central memory population, identified using the alternative phenotype CD45RO$^+$CD45RA$^+$CD62L$^+$ is lost in RA [46]. CCR7 (CD197) is a chemokine receptor mediating trafficking of lymphocytes between secondary lymphoid organs [75]. CCR7 is expressed on the vast majority of naive peripheral blood T cells that also express CD27 and CD62L [76]. In RA, naive and memory T cells express abnormal chemokines receptor patterns in relation with inflammation and inflamed tissue homing [71].

CD28null subset

CD4$^+$CD28null T cells are oligoclonal lymphocytes rarely found in healthy individuals younger than 40 years of age, but more frequently observed in the elderly and in patients with chronic inflammatory diseases. They are the most consistent biological indicator of immune aging in humans and predict immune incompetence [77]. Contrary to paradigm, CD4$^+$CD28null T cells are terminally differentiated effector memory cells, expressing phenotypic markers for tissue infiltration and damage [78]. They are functionally active and persist because of altered responses to apoptosis-inducing signals, notably Fas-mediated apoptosis [79]. These CD4$^+$CD28null T cells form large and long-lived clonal populations, with potent effector memory functions with regard to their proliferation and cytokine-secretion profiles (producing high levels of IFN-γ notably), exhibiting autoreactivity and cytolytic activity representing a functional specialization for killing [18,80,81].

Large expansions of CD4$^+$CD28$^-$ T cells have been reported in the blood of a third of RA patients [80,82]. However, only when patients are cytomegalovirus seropositive [83]. Such cytomegalovirus reactivity was most prominent in patients with low reactivity to other tested autoantigens, suggesting that they may not play a direct role in autoimmune disease [78]. The frequency of CD4$^+$CD28null T cells correlated with extra-articular involvement (nodules), but not with disease duration or severity of joint destruction [80]. These cells were however excluded from the joints in most patients [80]. The repertoire of CD4$^+$CD28null cells in RA was also grossly skewed with the presence of expanded clones, with a dominant usage of a single V-β 14 element [81,84]. The fact that this marked oligoclonality of CD4$^+$CD28null T cells was specific to RA patients initially suggested a pathogenic role for these cells. However, the preferential use of V-β 14 may more likely reflect the general HLA shaping of the T-cell repertoire in RA [84].

Killer Ig-like receptors (KIRs; KIR2DS2, KIR2DL2 and KIR3DL2) are expressed on CD4$^+$CD28null T cells in RA [85–87] bridging functions of the innate and adaptive immune systems. KIRs were successively acquired within each CD28null clone following the initial clonal expansion [88]. Increased frequency of CD4$^+$CD28$^-$ cells in RA patients was also associated with evidence of atherosclerotic changes including arterial endothelial dysfunction and carotid artery wall thickening [89]. CD4$^+$CD28null T cells express functional IL-12 receptor. Costimulation in the presence of IL-12 restored the expression of a functional CD28 as well as CD25 and CD40L in RA *in vitro* [90]. Finally, TNF-α was shown to induce the

transcriptional silencing of the *CD28* gene suggesting a direct link between inflammation and development of CD28null cells.

CD57+ T cells

Increased frequency of CD3+ T-cell populations expressing the accessory molecule CD57 was observed in patients with acquired immune deficiency syndrome [91]. CD3+CD57+ T cells are present in normal individuals and expand during aging. They show a distinct phenotype of advanced differentiated memory cells. The same clonal expansion was observed in both CD57+ and CD57- cells suggesting a common origin, which was also evidenced by a similar clonal T-cell cytokine profile (for IL-2, IL-4 and IFN-γ) [92]. Most CD3+CD57+ T cells are CD8+ T cells (80%) but CD4+CD57+ T cells are also present [93].

In RA patients, CD57+ T cells are found in blood, joint fluid, synovial membrane and bone marrow [94]. Their frequency in blood correlated with disease duration and presence of a significant population of CD4+CD57+ cells was also associated with the presence of rheumatoid factor [95]. CD4+CD57+, but not CD8+CD57+ T-cell frequency correlated with some measures of disease activity such as erythrocyte sedimentation rate [96]. CD4+CD57+ cells in RA present a restricted repertoire, suggesting a strong antigenic pressure in selecting clones expressing CD57 [95], in contrast to the TCR V-β usage of the CD8+CD57+ population which was restricted to the common RA repertoire [97]. A third of CD3+CD57+ T cells produced IFN-γ, whereas only approximately 3% produced IL-4 in RA [96]. Expression of CD28/CD57 on T lymphocytes is often reciprocally related [83]. In contrast to CD28null cells, CD57(+) T cells showed a greater susceptibility to apoptosis due to an increased production of active caspase-3, an increase expression of Fas and Fas-ligand (FasL) as well as CD3-ζ level but a loss of survivin [98]. CD3(+)57(+) cell leukemia is frequently observed in RA [94].

CD4+ T-cell polarization

Innate immunity responds to pathogenic microbes in a nonantigen-specific manner and does not result in immune memory. Adaptive antigen-specific immunity occurs in a second phase, orchestrated by dendritic cells presenting antigen to naive T cells. Naive T cells then undergo polarization towards different cell surface and cytokine expression patterns. This is achieved by programmed alterations of gene expression regulated by structural changes in chromatin (T-bet for Th1 and GATA3 for Th2) Although an over-simplification, the Th1/Th2 paradigm remains useful to understand progression along the road of T-cell differentiation particularly when the maintenance and irreversibility of these CD4+ states is not challenged [99] as opposed to the Th17 state (see below, [100]). The nature and strength of the costimulation signal delivered by dendritic cells also drives T cells towards immunity or tolerance by delivering polarizing or tolerizing molecules promoting effector or suppressive responses [101]. The sequential generation and balance of effector T-helper subset cells (of the Th1, Th2 and more recently of the Th17 types) and Treg is central to the development of autoimmunity. In an animal model of experimental lineage determination from a naive T-cell population, IL-17 production occurs first followed by a decline associated with the appearance of IFN-γ-producing Th1 cells. Regulatory T cells appeared during the recovery phase of the disease [102]. Autoimmunity was suggested to predominantly relate to Th1 cells and the production of IFN-γ [103] however, IFN-γ was shown to be protective in other models of autoimmunity. TNF-α was found to enhance Th1 polarization by acting on IL-12 production in antigen-presenting cells and the expression of the IL12R on T cells [104].

Th1/Th2 polarization in RA

The profile of cytokines expressed in the joints of RA patients is predominantly a Th1 profile. Th1 cell frequency and IFN-γ production are elevated in RA and remain so in clinical remission and following anti-TNF therapy [70,105]. This suggests a Th1-driven disease resulting from an imbalance between Th1 and Th2 cells and hence insufficient Th2 to downregulate inflammation. T-cell clones derived from RA blood cells were mainly of the stable CD4+Th1 type [106], whereas, the few CD4+ Th2 and CD8+ T-cell clones produced were unstable and shifting back to Th0 and Th1 or cytotoxic T cells (Tc1 and Tc2) respectively, suggesting incomplete commitment towards either subset.

Despite compelling evidence for RA being a Th1-driven disease, defective Th1 polarization in RA has also been reported. *In vitro* differentiation of the T cells of early RA patients (treatment naive) into either Th1 or Th2 revealed no alteration in the pattern of secreted cytokine, however severe impairment in differentiation was observed [107]. Th1 cells mostly produced IFN-γ with reduced IL-2 production and very

few double-positive IL-2/IFN-γ cells. Expression of T-bet mRNA was positively correlated with IFN-γ levels, but negatively correlated with C-reactive protein levels [108], and IFN-γ production from RA patients' blood cells was significantly reduced [109]. IL-18 was able to rescue this phenotype in synovial T cells but not in the blood. This suggests that in RA, a polarized Th1 immune response may be present in the synovium but is suppressed in the peripheral circulation.

IL-7 is another important cytokine for the early events leading naive T cells towards Th1 polarization [110,111]. We have associated low levels of circulating IL-7 with reduced thymic T-cell development and T-cell function in the periphery of RA patients [112,113]. High levels of IL-7 were also directly correlated with higher levels of IFN-γ but not TNF-α, TGF-β or IL-2 [114]. Using the expression between T-bet and GATA3 as surrogate measures of Th1 polarization, we also showed that IL-7 levels were significantly associated with T-bet but not GATA3 expression, suggesting that the peripheral Th1 polarization impairment in RA may possibly be related to low levels of circulating IL-7. On the other hand, Th2 polarization of naive T cells from RA patients showed no impairment [115]. However polarization conditions required the provision of exogenous IL-4 compared with healthy controls. Animal models have also shown that TNF-α favours the development of Th1 cells [104]. However, more recent work suggests it can do the same for Th17 cells. Neutralizing anti-IL-12 antibodies in the collagen-induced arthritis (CIA) model, before the onset of arthritis did not lower the incidence of arthritis, but dramatically attenuated the severity of the disease [116] associated with reduced IFN-γ, TNF and IL-6 levels. Blocking of TNF in CIA mice also showed efficient inhibition of disease [117], however a synergy with IL-12 blockade showed a greatly enhanced effect. In contrast, repeated administration of IL-4 to the CIA model resulted in a shift of the Th1/Th2 balance and significantly delayed onset. This did not affect severity of disease, but a decrease in TNF-α secretion by synovial cells was observed [118] suggesting that IL-4 works by downregulation of Th1 responses rather than upregulation of Th2 responses.

Th17 polarization

Over the past 5 years, a considerable amount of interest has been raised in the immunological community about new polarization subsets. $CD4^+$ T cells that preferentially produce IL-17A and IL-17F can be generated as a separate lineage, now termed Th17 [33,68,119]. Consequently, further subsets preferentially expressing particular cytokines were discovered (see below). Th17-secreting cells express the RORγt as a key transcription factor for their differentiation [120]. They were initially discovered in mice and later explored in humans. However, while the presence of Th17 was demonstrated *in vivo* in humans, their relevance to health and disease pathogenesis is only just being understood [101]. In addition to their contribution to host defense against extracellular bacteria and fungi [121], they are also thought to be pivotal in the development of autoimmune diseases such as RA under pathologic conditions [94,121–129]. Major differences exist between murine and human Th17 cells, most notably with respect to their mechanism of generation and their phenotype. In addition, the role and function associated with Th17 in mice may not be transposable to humans [101,124].

Studies in animals suggested a pathogenic role for Th17 cells in several autoimmune disorders and notably RA [130–133]. Antibodies blocking IL-17 reduced inflammation and bone erosion in CIA [134,135] and consistently, $IL\text{-}17^{-/-}$ knockout mice are also resistant to CIA [132]. However, whether human autoimmune disorders, including RA, are prevalently Th1- or Th17-mediated is still unclear as questioned recently by Annunziato and colleagues. Moreover, it is still unclear whether or not the pathogenic role attributed to Th17 cells in human immunopathology was prematurely inferred from animal studies [100,123].

Nonetheless, elevated IL-17 levels were found in RA, SLE and psoriasis patients [136], however Th17 cell frequency is not elevated in the blood of RA patients compared with healthy controls [105,137]. Increased Th17 cell frequency was observed in synovial fluids whilst frequency of tissue Th17 was no more numerous than in blood [137]. $CD14^+$ monocytes isolated from RA joints with an *in vivo* activated phenotype readily induced Th17 differentiation *in vitro* from memory $CD4^+$ T cells but not Th1 or Th2 [122]. Activation was TNF and IL-1β independent, in contrast to LSP-activated $CD14^+$ monocytes which required cell–cell contact. Antigen presentation-independent differentiation of Th17 cells using TLR-4- or TLR-8/7-conditioned media from stimulated RA patients peripheral blood mononuclear cells was also possible with IL-1β, IL-6 and IL-23 as essential inducers and TGF-β as the enhancer [138]. Memory $CD4^+$ T cells isolated from early RA patients (treatment naive)

and stimulated with anti-CD3/anti-CD28 could also produce higher amounts of IL-17, TNF-α, IL-22 and IFN-γ but low levels of IL-4 [139]. In the presence of VitD3 (1,25[OH] vitamin D-3) this stimulation was inhibited. Promoter gene regulation via epigenetic mechanisms was also demonstrated [140]. Furthermore, osteopontin produced by RA synovial fibroblasts specifically induced the expression of IL-17 by T cells independently of IL-6 stimulation [103]. The phenotype of synovial Th17 was altered compared with blood cells with higher expression of the TNF-R, but lower IL22-R and IL-23R and increased production of cytokines such as IFN-γ. The expression of CCR6 on Th17 cells also diminishes in remission [105].

In human tissue however, only a small proportion of IL-17 is produced by Th17 cells, as opposed to animal models of arthritis where IL-17 is mostly produced by T cells suggesting an alternative source of IL-17 production. Surprisingly, IL-17A expression was mostly colocalized within mast cells [141], where the production of IL-17 was conditioned by the expression of ROR-C in response to stimulation by TNF-α, IgG complexes or C5a. Fibroblasts have since been explored and, as for many other cytokines, have also been found to express IL-17 in RA synovial tissue. Nevertheless, IL-17 was shown to activate a number of pathways involved in recruiting immune cells to sites of inflammation by producing chemokines (notably CXCL1, 2, 5, 8, 9, 10 and CCL2, 20 [136]), enhancing inflammation through cytokine production (IL-6 and TNF-α) and acute phase response (serum amyloid A [125] and C-reactive protein [126]), and participating in tissue damage (MMP1, MMP3, MMP9, MMP13 and TIMP1) [127–129]. Most importantly, these led to significant bone loss mediated by osteoclasts [142]. Notably, the discovery of Th17-induced osteoclatogenic activity has been able to explain how bone damage can occur in the presence of IFN-γ which has long been known for its antiosteoclastogenic effect [143].

At the current time however, the role of the plasticity demonstrated between Th17 and other polarization subsets remains a major point to understand [100,144]. Th1 and Th2 cells are both in a stable state of differentiation, although some degree of overlap in their cytokine expression profile can be observed with repeated stimulation with the opposing cytokine triggers [145,146]. In contrast, the transient nature of the Th17 phenotype is now well established in humans and was thereafter further demonstrated in mice. Human Th17 cells also present features that are incompatible with the Th17 paradigm established in mice. For example, human T-cell clones often present a mixed phenotype termed Th1/Th17 as well as expressing markers for both subsets [147]. Of major importance was the discovery that in the presence of IL-12, human Th17 clones transform into Th1 cells, whereas Th1 cells do not, providing further evidence that human Th17 cells are unstable and can shift to a Th1 profile. Th17 clones could also be induced to produce IL-4 (Th17/Th2 cells), most notably in patients with asthma [148]. This high degree of plasticity inherent to the human Th17 phenotype is therefore becoming more apparent and the dynamic relationship between subsets is more complex than originally thought, questioning the initial assumption that Th17 cells are responsible for a number of autoimmune diseases.

Other polarization subsets

Subsets of CD4+ T cells able to produce IL-9 or IL-22 have recently been described in mice models and termed Th9 or Th22 [149–153]. IL-9 is however produced by other T-cell subsets (notably Th2, Th17) and a role in regulating pathogenic versus protective mechanisms of immune responses has been suggested due to the role of IL-9 in the differentiation of other subsets notably Th17 and Treg [151,153]. A specific transcription factor for Th9 polarization has not been identified. A putative role for Pu.1 [154] and the possibility that Th9 are a subset of Th2 cells have still not been fully discarded [155]. Several recent publications implicated Th9 in multiple sclerosis by analogy with animal models. To our knowledge, Th9 have not yet been investigated in RA. Human Th22 cells were differentiated from naive cells in the presence of IL-6 and TNF [152,156]. Several reports have identified Th22 cells in human, notably in psoriasis and psoriatic arthritis. Th22 cells were recently detected in RA patients in higher numbers than in osteoarthritis and healthy controls [157], furthermore, their frequency was correlated with disease activity and inflammatory markers (C-reactive protein). Blocking their differentiation or plasticity has therefore been proposed as potential therapeutic intervention [158]. Follicular T cells (Thf) were described a decade ago as cells present in tonsil and secondary lymphoid tissue [159,160]. These Thf cells are characterized by the expression of Bcl6 localized in the T cell/B cell zone of germinal centers [161,162], and appear to have an essential role in the provision of T cell help to B cells. To date, the role of Thf in RA synovitis has not been fully explored however, the presence of ectopic germinal centers has been described in

approximately one-third of patients [163]. The presence of high levels of IL-7 in the RA synovium may also contribute to the differentiation of such lymphoid structures [164,165].

Regulation & Treg

The concept of T-cell-mediated immune suppression has received renewed interest, following the association over a decade ago between high-expression levels of the cell surface marker CD25 and regulatory capabilities. This discovery enabled the identification, quantification, purification and analysis of naturally occurring Treg [166,167]. However, evidence of thymic-derived T cells acting as regulators in animal models of arthritis was reported in the late 1970s [168–170].

In humans, concavalin A-induced T-cell proliferation was shown to produce an autologous Leu-2$^+$ CD8$^+$ T-cell population with suppressive activity, defined as the ability to inhibit the response of fresh allogenic T cells when used in an *in vitro* mixed leukocyte reaction [171–173]. RA patients showed reduced suppressive capabilities by this population, which were however normalized in clinical remission and the presence of autoantibodies also appeared to decrease this suppressive activity [174,175]. No differences were observed between severe and less active disease [174]. This was the first suggestion that patients with active RA had abnormal suppressor cell function. The energy associated with RA was therefore investigated and associated with relatively normal nonspecific suppressive function, although individual patients could show strikingly abnormal profiles [176,177]. The ratio of CD4$^+$/CD8$^+$ T cells was then used to assess the loss of this regulatory population which was marked in SLE but less so in RA [178].

Further work demonstrated that induction of these CD8$^+$ suppressive T cells relied on the presence of a CD4$^+$ T-cell subset which expressed the homing receptor Leu8 [171]. Using the CIA model, the existence of a suppressive CD4$^+$ T-cell population with a memory phenotype was confirmed using transfer experiments [179]. Antibodies binding to Leu8 were also shown to cause Leu8$^+$ B cells to inhibit their immunoglobulin synthesis, however, inhibition was abrogated by the addition of lymphokines (now, called cytokines) [180,181]. Later with the advance of two-colour flow cytometry and the discovery and increased availability of monoclonal antibodies, a reduction in frequency of this suppressive Leu8$^+$CD4$^+$ T-cell subset in RA was shown to be directly related to disease activity (Lansbury's index) [182]. However the use of different methods made the field extremely controversial. Interestingly, response to the drugs used at this time correlated with normalization of this subset whereas in patients with disease which remained active a further loss was observed [182].

Naturally occurring Treg in RA

The CD4$^+$CD25high Treg phenotype is now widely recognized. Treg have been implicated in a number of conditions, including autoimmunity [183–188], tumor immunity [189,190], response to pathogens [191,192] and transplantation [193]. Quantifying Treg in human health and disease has therefore become an important issue, with a number of hypotheses based on whether or not Treg number and function are altered. Flow cytometry has been the method of choice for rapid quantification of the Treg subset, but in humans this has proven to be a challenge and a number of studies have reported conflicting data, with similar [194], increased [195] or decreased [196,197] circulating Treg frequencies in RA. Treg presence was demonstrated in synovial tissue and fluid [184] at a consistently higher frequency compared with blood [194,195,198], suggesting an active recruitment of Treg to the affected joint. No functional defect in circulating [195,199] and synovial cells [198] could be identified in terms of inhibiting proliferation, suggesting an intact capacity and allowing them to contribute to dampening of local inflammation. These data were challenged by other reports of reduced suppressive function for blood-derived Treg [200–202], thereby leaving us with conflicting data. Synovial fluid Treg showed enhanced suppressive capacity; however this was counterbalanced by the activated phenotype of T cells [195]. Cytokines produced in coculture suppression assays from RA synovial fluid and peripheral blood showed reduction in both Th1 and Th2 factors, including IL-17 [198]. Enhanced suppressive capabilities [195,203] and differences in Treg phenotype (expression of activation markers such as FOXP3, cytotoxic T-lymphocyte antigen 4 [CTLA-4], glucocorticoid-induced TNF-R [GITR], HLA-DR, CD69 and OX40 [194,195,203]) were however identified in RA synovial Treg. More recent work following the identification of additional markers, including Foxp3 and low expression of the IL-7R (CD127) [204,205], has not fully resolved these issues however, more consistent reduction in CD4$^+$CD25^{high}Foxp3$^+$CD127^{low} Treg frequency in RA patients is now reported compared with healthy controls [206,207].

Since studies of human Treg have relied on *in vitro* assays, potential interference by an inflammatory local microenvironment was not taken

into account. Synovial fluid Treg, with surface expression of TNF-RII and IL-7R, are susceptible to regulation by these cytokines. Treg functionality in RA was therefore abrogated in the presence of TNF-α, notably with regards to their ability to secrete cytokines. In humans, circulating Treg frequency correlated inversely with markers of bone resorption [208]. TNF-α has a direct effect on Treg's ability to inhibit osteoclast differentiation in mice [209] whilst the bone protection mediated by Treg was independent of the suppression of inflammation in the hTNF-Tg model [208]. *In vitro*, TNF-α abrogates the suppressive activity of Treg by reducing Foxp3 expression [202]. In contrast, IL-6 did not influence Treg-mediated suppression. We showed that IL-7 also abolished Treg activity, however our data suggest that this is mediated by increased effector function of T cells rather than by directly affecting Treg [Churchman SM *et al.*, Modulation of peripheral T-cell function by interleukin-7 in rheumatoid arthritis (2012). Manuscript in preparation].

When Treg were stimulated in the presence of IL-2/IL-15 they differentiated into IL-17-producing cells with high expression of the Th17-related transcription factor ROR-γt [196] and were positively identified by CCR6 expression [197]. IL-1β, IL-6, IL-23 and IL-21 or TGF-β enhanced this phenomenon [210]. This conversion required epigenetic remodeling to erase the Treg phenotype. This plasticity of Treg cells in RA may explain why they are not capable of suppressing responses at the disease site. Retinoic acid on the other hand was shown to promote Treg activity and prevent their conversion into Th17 cells [211,212], even in the presence of IL-6 [213].

Effects of therapies on T-cell subset in RA

Lessons from years of treating RA with T-cell targeted therapies should however, not be forgotten. Treatments that specifically deplete T cells (total lymphoid irradiation, thoracic duct drainage, high-dose chemotherapy with autologous stem cell transplantation or the AIDS disease) led to clear improvement in RA supporting the T-cell model [214]. Antibodies producing profound CD4+ T-cell depletion showed efficacy, but at the cost of excessive toxicity [215,216]. Nondepleting anti-CD4 antibodies that induce immunological tolerance [37] had short-term clinical benefit [217]. Tolerizing anti-CD3 antibodies also demonstrated short-term symptomatic improvement associated with an increase in regulatory T-cell numbers after treatment [112]. Progress in understanding T-cell tolerance and its modulation by costimulatory pathways, led to the development of new drug concepts, such as abatacept (CTLA-4-Ig) [218] and its successful use in RA [219]. Overall, these therapies directed at T cells have all showed clear clinical benefit but often with relatively short-term efficacy. The reasons for this failure are unclear but disease heterogeneity is likely to be a contributing factor. The inability to deplete or inactivate synovial T cells may be another explanation, and indeed, this raises the interesting point that T-cell trafficking of cells between joint tissue and the periphery is also an important consideration [220]. This is illustrated by the observation that following lymphodepletion, clinical response and relapse correlated with the depletion and return of CD4+ T cells (but not CD8+, B cells or macrophages) to the synovium [47,71].

Since the development of biologic drugs a decade ago, the effect of these therapies on T cells have been explored in RA patients for blocking or depleting antibodies and decoy soluble receptor. A direct effect of inhibiting TNF-α was the restoration of CD28 expression on T cells [89,221] confirming the relationship between presence of CD28null cells and exposure to chronic inflammation. Naive frequency was reduced early in RA progression however, the maintenance of a frequency close to age-matched controls at baseline was associated with the achievement of remission in patients treated with either methotrexate (MTX) or anti-TNF [Ponchel F *et al.*, Induction of remission in early RA can be predicted at baseline using T-cell subset analysis (2011), Submitted] [222]. Anti-TNF therapies in both early and established RA resulted in naive cell frequency increasing with time only in responders to MTX or biologics [222]. The inhibition of the spontaneous IFN-γ production and Th1 response was also abrogated and the low IFN-γ:IL4 expression ratio increased towards normal levels [223]. The low expression of Tbet was not affected by TNF blockade [114,223] as well as in remission induced by disease-modifying antirheumatic drugs [47,71,114]. In contrast, both the frequency of Th17 cells and IL-17 production also decreased following TNF blockade [105] in relation with the unstable nature of this subset. Relapse in patients achieving remission both on disease-modifying antirheumatic drugs [Ponchel F *et al.*, Unpublished Data] and on biologics [206] was best predicted by higher naive cell frequency as well as safe discontinuation of biologics drug [206]. The defect in cytokine expression in Treg was also corrected post anti-TNF therapy in association with a rise in the frequency of circulating Treg [200]. Our own data reproduced these results

in early RA showing a small increase in Treg ($CD4^+CD25^{high}Foxp3^+CD127^{low}$), which was not seen in MTX-treated patients [216] [PONCHEL F *ET AL.*, INDUCTION OF REMISSION IN EARLY RA CAN BE PREDICTED AT BASELINE USING T-CELL SUBSET ANALYSIS (2011), SUBMITTED]. In established RA, this increase was only transient. Other therapies also gave rise to an increase in Treg frequency in responders including IL-6 blockade [PONCHEL F *ET AL.*, UNPUBLISHED DATA], B-cell depletion [224] and recently, atorvastatin [225]. In contrast to these data, an increased frequency of $Foxp3^+$ T cells was associated with RA patients relapsing after achieving remission on TNF-blockade [206]. Foxp3 is also upregulated during T-cell activation [226] and refinement of the Treg phenotype using $CD4^+CD25^{high}Foxp3^+CD127^{low}CD62L^+$ cells was shown to associate higher Treg frequency with safe discontinuation of anti-TNF therapy in early RA [206].

Future perspective

Taken together, these findings provide substantial evidence for abnormal T-cell development and differentiation in RA, ranging from impaired thymic output to accelerated immuno-senescence, abnormal polarization as well as defective capability linked to the presence of proinflammatory factors in their microenvironment. Over the next 5 years, a greater understanding of these phenomena should not only improve our understanding of the disease, but may also suggest novel therapeutic avenues.

Executive summary

Evidence for roles of T cells in rheumatoid arthritis

- Beside the major histocompatibility complex-association (HLA-DRB1 notably) with rheumatoid arthritis (RA), infiltration of immune cells including T cells, B cells and macrophages in the synovial joint tissue and fluid remains the main feature of joint inflammation.
- Formation of extopic secondary lymphoid tissue like structure suggested to a T-cell centric model where T cells orchestrate the inflammatory response.
- However, the lack of common antigen, the nonproliferative nature of synovial T cell, the lack of T-cell-produced cytokine questioned this model in favour of a network of cellular response in established disease however, the driving role of T cells in very early disease remians an accepted fact.

General features of T-cells in RA

- Several genome-wide association studies suggest a list of approximately 30 loci related to T-cell pathways and T-cell interaction with other cells.
- Accelerated T-cell aging evidenced by telomeric shortening as well as compromised thymic activity due to IL-7 deficiency.
- T-cell repertoire distortion imposed by the HLA-DRB1 allele shaping and preferential selection of certain V-β and J-β segments affect both the naive and the memory repertoires.
- Abnormal signaling evidenced in several pathways: RAP1, Ca^{2+}, TCR-CD3, phosphorilation cascades (LCK and ZAP70) and Red-OX balance.

T-cell subsets

- Classic differentiation model is perturbed with cells presenting naive and memory propoerties.
- The CD28null subset considered a biological indicator of immune aging and a predictor of immune-incompetence are often raised.
- Presence of advanced differentiation memory CD57+ T-cells in nrelation with diseses activity.

$CD4^+$ T-cell polarization

- Th1 cytokine profile (notably IFN-γ production) suggested a Th1 driven disease.
- However, accumulation of evidence for defective Th1.
- Elevated IL-17 cytokine expression in synovil tissue and presence of Th17 cells.

Regulation & Treg

- Conflicting results related to the frequency of naturally occurring Tregs in RA due mostly to technical issues with the deficition of their phenotype and method of charaterization.
- Conflicting results as to their biological activity also due to difference of methodology.

Effect of therapied on T-cell subsets in RA

- Many treatment option have targeted T-cells clear clinical benefit but with short term efficacy.
- Clinical response (also to non-T-cell-targeted therapies) and relapse correlated with the depletion and return of $CD4^+$ T cells (but not CD8 T cells, B cells or macrophages) to the synovium.

Conclusion

- Evidence for abnormal T-cell development, signaling and differentiation.
- A greater understanding of T cells' biological response would improve our understanding of RA and suggest novel therapeutic avenues.
- The use of T cells for monitoring disease activity and predict response to therapy need to be explored further.

For example, the therapeutic use of Treg has shown promising data in animal models; however overcoming the technical hurdles regarding their purification and expansion without losing their capacities as well as controlling the microenvironment in which such cell-base therapy would be used (in order not to inhibit their function), remains an essential prerequisite. In addition, Treg specificity for an antigen has also been shown to be essential for the success of such therapies [227] and this remains elusive in RA. Much work is needed to assess whether citrullination for example could provide such specificity.

Similarly, if it were possible to define the stage at which RA T cells deviate from normal maturational pathways, it may also prove possible in the future to address this abnormality. Thus, the specific removal of subsets associated with pathological function (Th17 cells) would offer an alternative strategy, however this concept is limited by the identification of unique cell surface markers to enable targeting of therapies specifically to these cells. Limiting the triggers of development for such subsets may be more realistic and as such anti-IL12 and IL-23 may be relevant.

Progresses in monitoring disease and predicting response to therapy are also needed clinically and T-cell subset as biomarkers hold promises in this respect [206]. In the future, building an immunological picture of patient using dysregulation of T-cell subsets may prove potentially useful for guiding clinical decision. This is an important topic that we are actively pursuing.

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