IL-27 – a double agent in the IL-6 family

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Abstract–
The cytokine interleukin (IL)-6 is a major therapeutic target for the treatment of various inflammatory and autoimmune diseases. While IL-6 receives considerable attention in studies of innate and adaptive immunity, the IL-6-related family member IL-27 is increasingly recognized for its effects on cellular proliferation, differentiation and leukocyte effector functions. Both cytokines activate responses in myeloid and stromal tissue cells where they direct the transition from innate to adaptive immunity. However, they are frequently identified as lymphokines that control responses in T cells and B cells. In this regard, IL-27 often opposes the action of IL-6. Here, we will review the role of IL-6 and IL-27 in inflammation, with a particular focus on inflammatory arthritis, and discuss their importance in the diagnosis, stratification and treatment of autoimmune disease.

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The IL-6 family of cytokines –
All members of the interleukin (IL)-6 family share a common 130kDa glycoprotein signal-transducing receptor (gp130, CD130). In this regard, receptors for IL-11, oncostatin-M (OSM), ciliary neurotrophic factor (CNTF), cardiotoxin-1 (CT-1), leukemia inhibitory factor (LIF) and the cardiotoxin-like cytokine (CLC) all utilize gp130 to transmit cytokine responses. These IL-6-related cytokines are structurally related and each contain 4 long α-helical chains, which are arranged in an up-up-down-down topography [1]. In contrast, the IL-6-related cytokine IL-27 is a heterodimeric cytokine consisting of two independent subunits termed IL-27p28 (also known as IL-30) and EBI3 [2]. IL-27 therefore resembles IL-12 (which comprises IL-12p40 and IL-12p35), and the related IL-23 (IL-23p19 and IL-12p40) and IL-35 (IL-12p40 and EBI3) inflammatory cytokines (Figure-1) [3]. However, IL-27 also shares several characteristics common to the IL-6 cytokine family. First, the receptor complex for IL-27 contains the IL-27 receptor-α (IL-27Rα, also known as WSX-1 and TCCR) subunit together with gp130 [4]. While gp130 is universally expressed in all tissues and organs, IL-27Rα is mostly restricted to lymphocytes, monocytes and osteoclasts [1, 3]. The cognate α-subunit of the IL-6 receptor (IL-6R, CD126) also shows a similarly restricted pattern of expression and is found on hepatocytes, leukocyte subsets and megakaryocytes [5]. Second, EBI3 shares close sequence identity with IL-6R [3, 6]. In this regard, the soluble IL-6R when bound to IL-6 resembles a heterodimeric cytokine reminiscent of IL-12, IL-23, IL-27 and IL-35 [5-7]. Third, IL-6 and IL-27 receptor activation leads to signaling through the latent transcription factors Signal Transducer and Activator of Transcription-1 (STAT1) and STAT3 [8]. Interleukin-27 is however the only member of the IL-6-related cytokine family to predominantly signal via STAT1 instead of STAT3 [1, 3]. Consequently IL-6 and IL-27 elicit both common and distinct biological outcomes, and IL-27 can often limit IL-6/STAT3-driven events.

The inflammatory significance of IL-6 and IL-27 –
Based on the biological properties of IL-6, this cytokine was originally named interferon β2, hepatocyte stimulating factor, cytotoxic T cell differentiation factor, B cell differentiation factor and B cell stimulatory factor-2. These broad definitions identify IL-6 as a lymphokine and activator of the acute phase response [5]. However, clinical experience with the blocking anti-IL-6R monoclonal antibody tocilizumab has helped unearth roles for IL-6 in the control of lipid, glucose and iron metabolism, regulation of the neuroendocrine system, and changes in psychological wellbeing that include pain, fatigue, mood and depression (Figure-2) [5]. Thus, IL-6 often displays features of a hormone. In contrast, IL-27 is primarily associated with the control of innate and adaptive immunity to infection [3, 9]. Interleukin-27 was first recognized as a pro-inflammatory cytokine due to its ability to support the development of interferon (IFN)-γ secreting T-helper cells. For example, IL-27 promotes expression of IFN-γ, the transcriptional master regulator T-bet, STAT1 and IL-12Rβ2 [2, 8, 10-12]. These activities
closely resemble those of IL-12. However, subsequent studies have shown that IL-27 is a negative regulator of IL-2 and can restrict development of immune responses (Figure-3) [13-18]. For example, IL-27 is required for the development of T-bet+, CXCR3+ Treg populations following Th1-mediated inflammation using Toxoplasma gondii challenged IL-27R-deficient mice [14]. In this regard the anti-inflammatory properties of type-I interferons are largely attributed to the upregulation of IL-27 and the subsequent promotion of IL-10 [19-21]. Consistent with these observations, prominent immunosuppressive roles were discovered for IL-27 through investigations in mouse models of chronic infection and autoimmunity [22-26]. In the absence of a regulatory IL-27 signal, IL-27R-deficient mice developed profound or lethal T cell-mediated pathology [13, 16, 27, 28]. In this context IL-27 often antagonises the actions of IL-6. While IL-6 supports the development and expansion of T helper cell responses [5], IL-27 has emerged as an inhibitor of Th17 activities and in a model of helminth infection limits Th2 responses through inhibiting GATA3 expression (Figure-3) [8, 18, 29]. While additional investigations are required to fully explore the wider biological functions of IL-27, emerging data also highlight potential roles for IL-27 in the control of pain [30], myeloid cell activation [31-37] and stromal tissue responses (Figure-2) [38-40]. Thus, IL-6 and IL-27 contribute to inflammation and the regulation of both innate and adaptive immune responses.

Although the receptor complex for IL-27 signaling remains fixed, composed of IL-27Rα and gp130, the signaling mechanisms employed by IL-6 are highly complex (Figure-1), and it is often challenging to understand how IL-6 receptor signaling can elicit a diverse array of biological responses. Three very distinct forms of IL-6 receptor signaling have now been proposed. These are termed classical IL-6 receptor signaling, IL-6 trans-signaling, which is reliant on the presence of a soluble form of IL-6R (sIL-6R), and a newly reported mechanism called IL-6 trans-presentation [5, 41, 42]. In contrast, IL-27 uses a classical IL-27 receptor system based on the cellular expression of IL-27Rα and gp130. However, the IL-27p28 subunit of IL-27 has also been reported to antagonize IL-6-mediated T cell responses, and can potentially bind the IL-6R (Figure-1) [18, 43, 44]. So why does IL-6 adopt these different forms of signaling? Here, it is important to note that IL-6 contributions to both the regulation of immune homeostasis and inflammatory responses are relevant to infection, trauma or injury [5]. During health, classical IL-6 receptor signaling promotes the maintenance of normal physiology. For example, IL-6 controls various metabolic processes and tissue renewal or regeneration [5]. In contrast, IL-6 trans-signaling is more widely associated with the regulation of inflammatory processes relevant to disease [5, 41]. This distinction is not however black and white. In this regard, classical IL-6 receptor signaling controls both the acute phase response and the generation of certain effector CD4 T cell populations [5]. Similarly, IL-6 trans-signaling has been linked to processes including hematopoiesis and the sleep REM cycle [45, 46]. The newly described IL-6 trans-presentation mode of cell activation is a juxtacrine
mechanism of IL-6 signaling that promotes the engagement of dendritic cells with T cells [42]. While further work is required to establish the precise biological significance of IL-6 trans-presentation, this mode of cell activation may control immunological processes in tissues that rely on resident immune cells to mount an appropriate response to antigen challenge. These locations may include sites with immune privilege such the brain or eye.

In the accompanying sections, we will consider the roles of IL-6 and IL-27 in the progression of inflammatory disease and will focus on their involvement in rheumatoid arthritis.

The significance of genetic polymorphisms linked with IL-6 and IL-27 –
Several lines of genetic evidence support a role for IL-6 and IL-27 in autoimmunity, cancer and infection. Genome-wide association studies and analyses of genetic polymorphisms have identified several susceptibility loci relevant to IL-6 and IL-27 that predict a predisposition for autoimmune disease. For example, a single nucleotide polymorphism proximal to the IL6 (rs1800795) transcriptional start site is associated with an increased incidence of coronary heart disease, idiopathic juvenile arthritis and other inflammatory conditions [47-50]. Equally, genetic variants associated with IL6st (gp130; rs10940495) and IL6R (rs2228145) are common to patients with cardiovascular disease and rheumatoid arthritis [47-50]. Several polymorphisms linked with IL27 (encoding IL-27p28; rs153109, rs181206, rs17855750) also display risk susceptibilities with asthma, certain cancers, metabolic disorders and some viral infections [51-53]. For example, rs153109 is linked to more severe forms of rheumatoid arthritis [54]. While additional functional genetic studies are required to determine the biological relevance of these genetic variants, several contribute to changes in cytokine or cytokine receptor expression. For example, mutations within IL6R (rs2228145) and IL6 (rs1800795) contribute to elevations in circulating sIL-6R or IL-6, which reflected an altered risk of cardiovascular disease, enhanced susceptibility to insulin resistance, obesity, and other inflammatory complications [47-50, 55-57]. Comparable studies of IL-27 related polymorphisms require further investigation.

The therapeutic opportunities afforded by IL-6 and IL-27 –
The success of interleukin-6 inhibitors in rheumatoid arthritis and related conditions illustrates the prominent role this cytokine plays in the underlying pathology. There are now several biological drugs that target the cytokine itself (e.g., clazakizumab, olokizumab, vobarilizumab, sirukumab), the IL-6R (e.g., tocilizumab, sarilumab), or the soluble form of IL-6R (e.g., olamkicept) [5]. Some of these are now approved for the treatment of rheumatoid arthritis, systemic juvenile arthritis, polyarticular juvenile idiopathic arthritis, giant cell arteritis, and Castleman’s disease. In addition, Janus kinase inhibitors (e.g., tofacitinib, baricitinib, ruxolitinib) also impact IL-6 receptor signaling as part of their mode of action [5,
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41]. While these drugs are well tolerated and offer clinical benefit, the development of sirukumab was recently stopped following a negative review from the FDA. However, the anti-inflammatory properties of IL-27 suggests that an IL-27 supplementation intervention may offer an alternate therapeutic strategy. For example, studies in experimental models of inflammatory arthritis, and ex vivo culture systems show that IL-27 inhibits the expansion of IL-17 secreting CD4 T cells (Th17 cells), restricts the development of ectopic lymphoid aggregates, and can reduce the severity of joint damage and bone erosion [58-60]. No clinical trials have however been conducted to explore this approach in more detail.

A similar strategy was previously adopted to test the anti-arthritic properties of recombinant IL-11. However clinical trials with recombinant IL-11 in rheumatoid arthritis failed to reach clinical endpoint and were suspended [61]. The biology of IL-27, and its particular influence on adaptive immune responses (Figure-3), means that IL-27 may be a more attractive intervention therapy.

In rheumatoid arthritis, synovial IL-6 and IL-27 levels correspond with differences in disease activity [58, 62-64]. For example, IL-6 expression correlates with poor disease prognosis including elevated acute phase activity, fatigue and increased cardiovascular risk. In contrast, synovial IL-27 levels correspond with a reduction in IL-17 and IL-6, and the Th17 chemoattractant CCL20 [62]. Thus, elevated levels of synovial IL-27 in inflamed rheumatoid arthritis joints may reflect an effort to counteract a persistent adaptive immune response. These findings are also reflected by studies in mice. Histological assessments of joint synovitis revealed that local IL-27-treatment resulted in suppressed leukocyte infiltration, synovial hyperplasia, cartilage and bone erosion, vascularization, and IL-6 and IL-17 expression in inflamed joints [59]. Systemic administration of IL-27 during collagen-induced arthritis also reduced type II collagen-specific antibody titers and serum levels of IL-17 and IL-6 [58]. Analysis of the peripheral immune CD4 T cell response also revealed that IL-27 inhibited the generation of IL-17-producing collagen-specific T cells, but promoted an increase in IL-10 secreting CD4 T cells and suppressive regulatory T (Treg) cells that regulate the expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) [65]. Interestingly, mice lacking IL-12p35 also develop a mild form of antigen-induced arthritis. This was attributed to an increased production of IL-27 and IL-10, and the expansion of Treg cells [66]. In this regard, IL-12p35 acts to suppress the action of IL-27 during inflammatory arthritis, and blockade of IL-27 activity was shown to restore the severity of synovitis [66]. The above studies highlight the therapeutic potential of IL-27 for the treatment of inflammatory arthritis and other diseases associated with autoimmune T cell-mediated pathology.

IL-6 and IL-27 in inflammatory arthritis –

Interleukin-6 is the archetypal member in the IL-6-related cytokine family. While many of these factors elicit similar biological responses in vitro, IL-6 often displays an over-riding influence on the same
Experiments in animal models show that IL-6-deficient mice are protected from various forms of disease [5, 41, 67]. For example, the induction of inflammatory arthritis in IL-6 deficiency is associated with an absence of synovial infiltration, synovial hyperplasia and joint damage (reviewed in [67]). This is not true for other cytokines in the IL-6 family. In models of arthritis, IL-11 Receptor-α (IL-11Rα)-KO mice and OSM Receptor-β (OSMRβ)-KO mice develop disease severity comparable to wild type controls [68]. However, there may be a context dependent caveat to this generalization [69-72]. For example, interleukin-11 regulates many anti-inflammatory outcomes in arthritis, which fuelled the aforementioned clinical trials with recombinant IL-11 [61, 73, 74]. The relationship between IL-6 and IL-27 is however different. In models of inflammation, IL-27 deficiency contributes to a more active pathology that includes the development of a more severe form of synovitis, and enhanced adaptive immune responses that are reflected by an increase in effector CD4 T cell numbers and antibody responses [60]. Thus, IL-6 and IL-27 acting via a common signaling receptor subunit elicit contrasting inflammatory outcomes that influence the initiation, maintenance and severity of joint pathology. While these activities primarily pertain to the control of adaptive immunity, both cytokines have influences on stromal tissue responses to inflammation. For example, IL-6 and IL-27 play important roles in bone remodeling, where an imbalance between bone resorption and formation contributes to bone destruction in inflammatory arthritis [75-78]. Here, Il27ra deficient mice with experimental arthritis displayed severe synovitis and synovial hyperplasia, and an increased incidence of focal bone erosions [60]. Systemic delivery of IL-27 reversed the development of these inflammatory parameters and inhibited osteoclastogenesis [79]. Notably, the action of IL-27 on inflammation-driven bone destruction can be both direct and indirect. For example, IL-27 abrogates RANKL responsiveness in osteoclast precursors and suppresses signaling downstream of RANK [36]. Interleukin-27 has also been shown to inhibit the production of RANKL in activated CD4+ T cells [80]. The impact of IL-27 on bone turnover is however not unexpected, and other IL-6 related cytokines including IL-11, OSM, LIF and CNTF also control aspects of bone homeostasis [81-84]. In summary, both IL-6 and IL-27 contribute to the control of synovitis and associated changes in cartilage and bone erosion.

**IL-27 suppresses synovial ectopic lymphoid-like structure development** –

Interleukin-6 and IL-27 are both lymphokines that control the survival, proliferation and effector characteristics of T cells and B cells and are thus poised to shape adaptive immune responses within inflamed joints. IL-6 has long stood as a key mediator in the generation of antibody responses and the formation of germinal center reactions [85, 86]. Recent studies have also highlighted the importance of B cell-derived IL-6 in promoting class-switch recombination of autoantibodies and spontaneous germinal center formation that are required for establishing systemic lupus erythematosus in mice [87].
While IL-27 has also been shown to drive the secretion of IL-21 in T follicular helper cells and support germinal center function [88], the IL-27p28 subunit can counteract IL-6-driven antibody responses and inhibit germinal center development [43]. Consistent with these roles, overexpression of IL-6 and the IL-6R in mice results in spontaneous inflammation featuring the formation of lymph node-like structures in the lung [89]. Similar lymphocytic aggregates called ectopic lymphoid-like structures (ELS; also called tertiary lymphoid structures, TLS) are a histopathological hallmark of tissue inflammation in a number of autoimmune diseases, cancers and infection [90]. New approaches such as ultrasound-directed small-needle synovial biopsy, combined with histopathological assessment of joint inflammation, has provided new insight into disease heterogeneity in rheumatoid arthritis [91]. Here, based on cellular and molecular signatures, synovitis can be classed into three pathotypes called ‘follicular’, ‘diffuse’ and ‘pauci-immune’. While diffuse pathology is characterized by a typical random infiltration of leukocytes primarily composed of macrophages and some T cells, the follicular pathotype features highly-organized and segregated aggregates of T and B cells accompanied by CD21+ follicular dendritic cell networks, active germinal centers and high endothelial venules (HEV). ELS are associated with the local priming of immune cells and autoantibody responses [92, 93].

Notably, our recent evaluation of IL27 and EBI3 expression (encoding IL-27p28 and EBI3 respectively) in the diffuse synovial pathotype mirrored previous studies that identified heightened levels of IL-27 in rheumatoid arthritis joint tissues as compared with control osteoarthritis joints [60]. However, compared to patients with diffuse pathology, the follicular form of disease was associated with reduced expression of IL27. Notably, IL27RA was highly expressed in the follicular form of rheumatoid arthritis, and cells expressing the IL-27R were localized at ELS. These observations suggest that distinct cytokine networks govern the development of synovial pathotypes, and that the absence of a regulatory IL-27 signal may contribute to the development of a follicular form of disease that is linked with severe local and peripheral inflammation and inferior responses to biological therapy (e.g., anti-TNF) [94-96].

Early investigations into the endogenous role of IL-27 in inflammatory arthritis revealed a pro-inflammatory role in proteoglycan-induced arthritis [97]. While this observation in IL-27R-deficient mice appears to contradict the therapeutic effect that has been observed following treatment with IL-27 in experimental arthritis [58, 59, 65, 79, 98], this may reflect the importance of a robust Th1 cell response for driving the proteoglycan-induced arthritis model. Our studies using IL-27R-deficient mice in the mBSA antigen-induced arthritis model revealed that these mice develop exacerbated joint inflammation, synovial hyperplasia, and cartilage and bone erosion that was accompanied by elevated peripheral Th17 cell and mBSA-specific antibody responses [60]. Reflecting the observation that IL27 expression was reduced in synovial biopsies from rheumatoid arthritis patients with a follicular-rich
form of disease, IL-27R-deficient mice developed synovial ectopic lymphoid-like structures that were associated with the expression of homeostatic cytokines (e.g., Lta, Ltb) and chemokines (e.g., Cxcl13, Ccl21) [60]. Thus, while IL-6 can promote ELS development in inflamed tissues, IL-27 is a negative regulator of ELS. An inhibitory role for IL-27 at ELS is consistent with observations in other models of inflammation that have linked heightened Th17-type effector responses (e.g., elevated expression of IL-17, IL-17F, IL-22, IL-21) with ELS development [99-101]. ELS development in antigen-induced arthritis was associated with the local expression of IL-17 and IL-21 [60], and effector cytokines linked with the Th17 programme (e.g., IL-17F, IL-21, IL-23, IL-22) have also been implicated in synovial lymphoid neogenesis in clinical rheumatoid arthritis [102]. The inhibitory control of effector Th17-type responses by IL-27 may therefore offer opportunities to identify new therapeutic targets for the treatment of the follicular form of rheumatoid arthritis.

Concluding remarks—

Cytokines that signal via the Jak-STAT pathway are increasingly viewed as therapeutic targets for the treatment of autoimmune diseases, infection and cancer. These include drugs that block IL-6 (e.g., olokizumab, clazakizumab, tocilizumab), IL-12p40 (e.g., ustekinumab), IL-21, IL-23p19 (e.g., risankizumab, guselkumab, tildrakizumab, mirikizumab) or GM-CSF (e.g., mavrilimumab) signaling, or members of the Jak protein family (e.g., tofacitinib, baracitinib, ruxolitinib). When considering the immuno-modulatory or anti-inflammatory properties of IL-27 it is tempting to consider how IL-27 intervention would supplement these therapies. Here, the capacity of IL-27 to inhibit Th17 development, and to promote the expression of checkpoint regulators and Treg activity, mirrors the therapeutic responses linked with tocilizumab treatment [3, 5]. However, further investigations are required to assess the context-dependent inflammatory activities of IL-27. These may require clinical trials in humans. While primary clinical endpoints will undoubtedly fixate on improvements in local tissue inflammation and damage, the wider implications of systemic inflammation are becoming equally important. For example, a metabolic shift associated with the systemic activation of T cells in PD-1-deficient mice was recently shown to impact the generation of brain monoamines and changes in emotional behavior [103]. In this respect, the bioactivity of IL-27 is interesting since IL-27 promotes the expression of PD-1 ligand PD-L1 [104]. Thus, an IL-27 intervention may offer opportunities to explore whether IL-27 can bring about improvement in disease activity and patient wellbeing. Such strategies would be relevant to clinical indications where IL-17 or Th17 driven outcomes promote disease progression (e.g., psoriasis). The question is whether supplementation with recombinant IL-27 can be used as a standalone intervention or an adjunct therapy in conditions where biological drugs that target IL-6, IL-12, IL-17 or IL-23 are effective. Several of the benefits associated with IL-6 blockade relate to the
impact of therapy on altered metabolic processes (e.g., anaemia through altered iron metabolism), fatigue and patient wellbeing. It is unclear whether recombinant IL-27 would elicit similar outcomes.

In summary, IL-6 and IL-27 appear to work in coordinated fashion, with IL-27 often suppressing the action of IL-6. These differences in biological activities reflect changes in the control of transcription factors STAT1 and STAT3 and may also relate to differences in the cytokine receptor subunits. While further work is required to fully appreciate the associations between IL-6 and IL-27, the current data offers interesting perspectives on how an IL-27 intervention may supplement existing biological drug therapies against IL-6, or members of the IL-12 cytokine family.

Acknowledgements –
GWJ and SAJ are supported by an Arthritis Research UK Career Development Fellowship (reference 20305) and programme grant (reference 20770) respectively. DGH is supported by a Medical Research Council PhD studentship and Life Science Research Network Wales, a research initiative funded through the Welsh Government’s Sêr Cymru program.

Competing interests –
SAJ has received funding support from Hoffman la Roche, GSK, Ferring Pharmaceuticals and Novimmune SA, and during the last 5 years he has acted as an advisory consultant for Roche, Chugai Pharmaceuticals, Novimmune SA, Genentech, Sanofi Regeneron, Johnson & Johnson, Janssen Pharmaceuticals, Eleven Biotherapeutics and UCB. GWJ has received funded for GSK and undertakes collaborative research with Medimmune. DGH and ACF declare no competing interests.
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**Figure Legends-**

**Figure-1 – The biological relationship between IL-6 and IL-27**
The illustration shows the composition of the IL-6 and IL-27 receptor complexes, and identifies the Signal Transducer and Activator of Transcription (STAT) factors triggered by both cytokines. Note the inclusion of gp130 in both receptors, and preferential induction of STAT1 and STAT3 activity (Bold text). For the IL-6 receptor cassette the reader should note the various IL-6 and IL-6R blocking therapies currently in clinical development or clinical utility. Proteins displayed in the orange box indicate biological entities that have been reported to engage with the IL-6 receptor, albeit at low affinity. Cytokines listed in the blue box showcase the protein composition of IL-27 related heterodimeric cytokines. Common subunits are colour coded. The IL-6:SL-6R (and that of p28:SL-6R) complex is not however stable and the cytokine-receptor undergoes association and re-association (indicated by the + symbol).

**Figure-2 – The functional properties of IL-6 and IL-27**
The biological properties of IL-6 and IL-27 have been broadly categorized under the terms ‘Inflammation’, ‘Homeostasis’, and ‘Wellbeing’. Defined activities have been listed for each category and the heatmap identifies the relative contribution of IL-6 and IL-27 to each of these processes. The definition of the colour coding is listed. It should be noted that IL-6 and IL-27 may regulate similar or distinct outcomes in each process and the reader is referred to the manuscript text and review articles relevant to IL-6 or IL-27 (references [3,5]).

**Figure-3 – Immuno-modulatory action of IL-27 and the interface with IL-6**
IL-27 and IL-6 together coordinate adaptive immune responses, often with opposing biological outcomes. In an inflammatory microenvironment, and supported by accessory cytokines, IL-6 can promote the differentiation of Th1, Th2, Th22 and Th17 cells. In contrast, IL-27 counteracts the IL-6-driven expansion of Th17 cells and inhibits the development of Th2 and Th22 cells. However, IL-6 and IL-27 can both promote the secretion of IL-10 in a number of effector T cell subsets, and can drive the production of IL-21 in T helper cells. IL-27 drives immunosuppressive effector characteristics in T cells including the expression of the immune checkpoints PD-L1, PD1 and CTLA4. In contrast to the inhibitory action of IL-6 on Treg cells, IL-27 promotes the development of IL-10-producing T-bet+CXCR3+ Treg cells and Tr1 cells. IL-27 also has immunosuppressive roles at the DC:T cell synapse, for example through promoting expression of PD-L1 on DCs and inhibiting MHC-I expression. **Boxed areas highlight opposing roles of IL-27 and IL-6.** Figure adapted from Yoshida et al. (reference [3]).