EVE DIAGNOSTICS

COMPREHENSIVE IMMUNE PROFILE (CYTOKINE, CHEMOKINE AND GROWTH FACTOR 71-PLEX) ANALYTE GLOSSARY

GROUP A – INNATE / AUTOIMMUNE INFLAMMATION

FGF-2 (Basic fibroblast growth factor)[1] – Growth factor that plays a role in the development and homeostasis of several tissues, notably in the musculoskeletal and cardiovascular systems, and is a pro-angiogenic factor. FGF-2 also has pro-inflammatory functions and its expression is increased in inflammation. FGF-2 may contribute to the pathogenesis of autoimmune conditions (i.e., RA and multiple sclerosis), colitis, orbitopathy in Graves disease, lung and kidney fibrosis, and cancer.

IFNα2[2] – A type I interferon, IFNα2 is expressed following viral infections and the recognition of stress-associated molecular patterns. IFNα2 induces an innate antiviral response preventing viral spread, and contributes to adaptive immune responses, notably type 1 immunity. There is evidence that IFNα2 expression may be detrimental in bacterial, fungal and parasitic infections, which may play a role in secondary bacterial infection following a primary viral infection. Increased expression of IFNα2 has been implicated in the pathogenesis autoimmune diseases like systemic lupus erythematosus and Sjögren's syndrome, and in autoinflammatory interferopathies.

IL-1α[3,4] A potent pro-inflammatory cytokine of the innate immune system and inducer of several components of the acute phase response. IL-1α is constitutively expressed in non-myeloid cells (e.g., keratinocytes, lung and gut epithelial cells) and is released from necrotic cells to initiate inflammation following tissue damage. IL-1 is also an important factor in the proliferation and function of Th17 cells, acting synergistically with **IL-23** to induce the release of **IL-17A**. Increased release of IL-1 has been observed in many pathological conditions, including autoimmune diseases, metabolic syndromes, acute inflammation, chronic inflammation, and invasive cancers. Studies in mouse models suggest that IL-1α, and not IL-1β, may be specifically implicated in inflammatory diseases of the skin, such as hidradenitis suppurativa and pyoderma gangrenosum, and in colitis.

IL-1β[3,4] Similar functions to IL-1α (both are ligands of a common receptor (IL-1R) with identical affinities). Unlike IL-1α, IL-1β is expressed only in myeloid cells and requires proteolytic processing prior to secretion to be activated. Activation is mediated by caspase-1 following the formation of inflammasome complexes upon detection of signals indicating the presence pathogens, inflammation, or physiological stress. IL-1β also supports the production of type 3 cytokines **IL-17A** and **IL-22** in ILC3s.

IL-2[5] **–** A key factor in Th1, Th2, Treg, and CD8+ T cell proliferation and clonal expansion (but inhibitory of Th17 development), characterized by an initial peak at the onset of the T cell response followed by a rapid decline. Low, baseline levels of IL-2 promote immunomodulatory Treg development and survival, whereas higher levels in response to immune stimulation promote the activation and proliferation of natural killer (NK) cells and CD8+ cytotoxic T cells. IL-2 has context-specific dual roles in either promoting or suppressing autoimmunity[6].

IL-17A[7] – A signature cytokine released by Th17 cells and one of the main drivers of type 3 immune responses, IL-17A contributes to host defense and pathogen clearance of extracellular bacteria and fungi, as well as regulation of the gut microbiota. Dysregulation of Th17 activity is a key driver of autoimmunity, and elevated IL-17A has been recognized as an important contributor to autoimmune disorders including psoriasis, rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease. IL-17A blocking agents

have been approved or are being investigated for the treatment of psoriasis, rheumatoid arthritis, ankylosing spondylitis, and multiple sclerosis.

IL-17E/IL-25[8] – An epithelial alarmin released in response to danger signals, IL-17E/IL-25 amplifies type 2 immune responses by promoting the production of **IL-4**, **IL-5**, and **IL-13** by Th2 cells and ILC2s, playing a contributing role to allergic inflammatory diseases such as asthma. It also promotes the pathogenesis of psoriasis, and may have both pro- and anti-inflammatory roles in inflammatory bowel disease. IL-17E has been shown to block Th17 polarization and to generally have a protective role in Th17-driven autoimmune disorders[9].

MIP-1α (Macrophage inflammatory protein-1α; CCL3)[10] – Recruitment of T cells, B cells, DCs, neutrophils, monocytes, and eosinophils to sites of inflammation via the receptors CCR1, CCR4 and CCR5. MIP-1α is an important factor in CD8+ memory T cell development in the lymph nodes and may promote Th1 polarization (and prevent Th2 polarization) in naïve CD4+ T cells[11]. MIP-1α has also been identified as a primary driver of progressive disease in multiple myeloma.

The analytes in this group are mainly associated with type 1 (Th1-mediated; IFNα2, IL-2, MIP-1α) or type 3 (Th17-mediated; IL-17A, IL-1) immune responses, so high levels of these markers could indicate infection with intracellular or extracellular pathogens, or autoimmune inflammation. IL-1, type I interferons, IL-17, and FGF-2 are known contributors to autoimmune disorders, and elevated IL-2 promotes active inflammation. IL-17A and FGF-2 have been shown to synergistically promote inflammation in autoimmune arthritis[12]. IL-1, IL-17 and FGF-2 can initiate and potentiate type 3 (Th17-mediated) immunity (a key driver of autoimmunity), while IL-2 and IL-17E/IL-25 have been shown to be negative regulators of Th17 activity. Type I IFNs have been shown to suppress Th17 cells in some contexts[13], although there is evidence that IFNα2 can exacerbate Th17-mediated inflammation in autoimmune diseases[14], such as in systemic lupus erythematosus (SLE) where IFNα2 and IL-17A contribute to a pathogenic signaling axis[15] . IL-1 is also one of the major drivers of innate inflammatory responses.

GROUP B1 – PRO-INFLAMMATORY CYTOKINES

Fractalkine (CX3CL1)[16] **–** Drives the recruitment of CX3CR1-expressing immune cells, including monocytes, macrophages, microglia, Th1 cells, CD8+ T cells, NK cells, γδ T cells, and conventional dendritic cells (cDC) to sites of inflammation. Fractalkine signaling contributes to continuing type 1 immune responses, and can also contribute to Th2 responses in asthma and Th17 responses in gut inflammation. The fractalkine-CX3CR1 axis has been implicated in a variety of diseases, such as atherosclerosis, allergic airway disease and asthma, inflammatory bowel disease, and cancer.

GM-CSF (Granulocyte-macrophage colony-stimulating factor)[17,18] **–** A pro-inflammatory cytokine that acts as a bridge between T cells and effector leukocytes during inflammation. GM-CSF also acts as a hematopoietic growth factor that drives the replication, differentiation, activation, and survival of a variety of myeloid cells and promotes pathogenic macrophage polarization. Most GM-CSF released during acute inflammation is released by CD4+ T cells (Th1 and Th17) and ILC3s, and contributes mainly to type 1 and type 3 immune responses, but can also play a role in type 2 immune responses in the airway. GM-CSF has been shown to be a major contributor to the pathogenesis of several chronic inflammatory and autoimmune diseases including rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and allergic diseases such as asthma.

IFNγ[19] **–** The major signature cytokine secreted by Th1 cells, as well as NKs, ILC1s, and antigen-presenting cells like DCs and macrophages, IFNγ is the key factor in orchestrating type 1 adaptive immune responses against intracellular pathogens. IFNγ promotes Th1 cell differentiation, induces M1 polarization in macrophages, drives the expression of anti-microbial and anti-viral factors, and regulates inflammatory responses by inducing several cytokines and chemokines (e.g., **IP-10** and **MIG**). As a key regulator of type 1 immunity, IFNγ dysregulation contributes to autoimmune and other chronic inflammatory conditions driven by Th1 cells.

TNFα (Tumour necrosis factor α)[20] – Important pro-inflammatory cytokine that is released following the detection of pathogen and other danger-associated molecular patterns, inflammatory signals, and stress, with a particularly important role in driving the early phase of the inflammatory response. TNFα is also released by Th1 and Th17 cells and has been shown to promote type 1, type 2, and type 3 immune responses, and is co-stimulatory in B cell development. TNFα is a pro-angiogenic factor that contributes to wound healing responses. Dysregulated production of TNFα has been associated with rheumatoid arthritis, inflammatory bowel disease, ankylosing spondylitis, psoriatic arthritis (PsA), juvenile idiopathic arthritis (JIA) and cardiovascular disease. TNFα blocking agents have been approved for the treatment of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, psoriasis, ulcerative colitis, and Crohn's disease.

> *The analytes in this group are potent pro-inflammatory cytokines that can initiate and propagate innate inflammation and contribute to adaptive immune responses. These analytes are all associated with type 1 immune responses – IFNγ, GM-CSF and TNFα are all produced by Th1 cells and NK cells, and fractalkine can amplify polarized type 1 responses and induce the recruitment of Th1 and NK cells*[21]*. TNFα, GM-CSF and fractalkine can also contribute to type 2 and type 3 immune responses in certain contexts.*

GROUP B2 – T HELPER CELL-MEDIATED INFLAMMATION

IL-4[22] **–** A key cytokine orchestrating type 2 immune responses, IL-4 promotes the differentiation, proliferation and survival of Th2 cells and is also secreted by activated Th2 cells. IL-4 induces Ig isotype switching to IgG1 and IgE in B cells and drives the maturation and activation of DCs. In the context of dysregulated Th2 immunity, IL-4 is thought to be a key factor in the initiation phase of allergic inflammation and related diseases (asthma, atopic dermatitis, etc.). IL-4 (with TGF-β or IL-1β) also drives the differentiation of **IL-9** producing Th9 cells.

IL-5[22] **–** A signature cytokine secreted by Th2 cells, IL-5 stimulates antibody production in B cells and is an important factor in the differentiation of mature eosinophils from their precursors. As a key factor in Th2-mediated responses, IL-5 contributes to diseases of allergic inflammation such as asthma.

IL-9[23,24] – A pleiotropic cytokine that is highly expressed by Th9 cells, mast cells and type 2 ILCs, and can be expressed by Th17 cells. Depending on the immunological context, IL-9 contributes to the regulation of Th1, Th2, and Th17-mediated immune responses and Treg-mediated immune tolerance, as well as regulating innate immune cells like mast cells, macrophages, ILCs, and dendritic cells. High levels of IL-9 have been associated with allergic diseases (e.g., asthma, allergic rhinitis, food allergies) and autoinflammatory/autoimmune diseases (e.g., inflammatory bowel disease, multiple sclerosis, lupus nephritis).

IL-12p40[25] **–** A subunit of the biologically active cytokines **IL-12p70** (heterodimer with IL-12p35, associated with type 1 immune responses) and **IL-23** (heterodimer with IL-23p19, associated with type 3 immune responses). IL-12p40 is highly expressed by macrophages and DCs upon microbial stimulation. When expressed at high levels, IL-12p40 can form homodimers that provide negative feedback to IL-12 and IL-23 signalling by competitively binding to the IL-12 and IL-23 receptors.

IL-12p70[26] **–** The active form of IL-12. Released mainly by DCs, monocytes, and macrophages upon bacterial- or viral-specific pattern recognition receptor activation, IL-12 is the main driver of Th1 cell differentiation and, when present with **IL-18**, induces **IFNγ** release from Th1 cells. Also promotes the differentiation of ILC1s and the proliferation of peripheral T cells and NK cells. IL-12 signaling is implicated in several inflammatory and autoimmune diseases, such as inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, diabetes mellitus, and Sjögren's syndrome.

IL-13[22] **–** A signature Th2-released cytokine that is also produced by CD8+ T cells, mast cells, eosinophils DCs, and ILC2s, IL-13 contributes to the type 2 immune response by serving as a co-stimulator that drives B cell maturation, activation and antibody release. IL-13 has been shown to inhibit the production of pro-inflammatory cytokines (**IL-1β, TNFα, IL-12**) by monocytes and may have a protective effect in type 1

inflammation, although both Th1 and Th17 cells have been shown to co-express IL-13 with their signature cytokines in some contexts[27]. IL-13 contributes to allergic inflammatory responses and is thought to drive the effector, rather than the initiation, phase of allergic diseases such as asthma.

IL-22[28] – A cytokine released primarily by lymphoid cells, including Th22 cells (thought to be the major source of IL-22 in the peripheral circulation), as well as Th17 cells, type 3 innate lymphoid cells (ILC3s), and NKs. Depending on the tissue and pathological context, IL-22 can either reduce inflammation and drive epithelial repair and regeneration, or it can induce pro-inflammatory mediators and promote type 1 or type 3 inflammation. High plasma levels of IL-22 have been observed in patients with inflammatory bowel disease, rheumatoid arthritis and malignancy.

MCP-3 (Monocyte chemotactic protein 3; CCL7)[29] – Induced in response to pathogen detection and inflammatory cytokine signaling (e.g. **IL-1β, TNFα, IFNγ**), MCP-3 signals via CCR1, CCR2, CCR3, and CCR4 to drive the recruitment of a variety of immune cells, most prominently monocytes, but also eosinophils, basophils, DCs, NK cells, Th2 cells, and neutrophils. MCP-3 signaling impacts the immune response to viral, bacterial, and fungal infections.

TNFβ (Tumour necrosis factor β; Lymphotoxin-α)[30] – Pro-inflammatory cytokine that also contributes to the development of the immune system. TNFβ is released by Th1 cells, CD8+ T cells, NK cells, and macrophages, and contributes to type 1 immune responses. It is also essential in the differentiation of NK cells and plays a role in NK recruitment and function. TNFβ has been implicated in the pathogenesis of rheumatoid arthritis (and may be a key factor in patients that are resistant to TNFα blockade), and cardiovascular disease.

> *The analytes in this group are largely associated with CD4+ T-helper cell mediated responses. The cytokine profile may indicate responses orchestrated by Th1 (IFNγ, IL-12p70, TNFβ; intracellular pathogen infection/autoimmunity), Th2 (IL-4, IL-5, IL-13, IL-9; helminth infection/allergy), and/or Th17 cells (IL-22; extracellular pathogen infection/autoimmunity) along with Th9 (IL-9*[24]*) and Th22 cells (IL-22, IL-13), which may contribute to in allergy and autoimmunity. Patterns of mixed T cell cytokine release could reflect the heterogeneity and plasticity observed in T cells in vivo, since "hybrid" cells that co-express the signature cytokines of two different T cell subtypes (e.g., IL-4 with IFNγ, IFNγ with IL-17A, IL-4 with IL-17A, IL-13 expressed by both Th1 and Th17 cells) may be common*[27,31,32]*.*

GROUP B3 – INNATE INFLAMMATION / CYTOKINE 'STORM'

BCA-1 (B cell-attracting chemokine 1; CXCL13)[33,34] **–** Drives the recruitment and promotes the differentiation of B cells and T_{FH} cells (along with small numbers of dendritic cells, NK cells, and macrophage progenitors) via CXCR5, playing a key role in the generation of lymphoid follicles. High levels of BCA-1 are associated with aberrant B cell and/or T_{FH} cell aggregates in several autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome, multiple sclerosis, etc.) and the initiation and progression of lymphoproliferative and solid tumour cancers. BCA-1 has also been identified as a predictive marker of lethal COVID-19 cases.

Flt3L (FMS-related tyrosine kinase 3 ligand)[35] – Hematopoietic growth factor that drives hematopoietic stem cells towards a lympho-myeloid fate, is essential in the generation of DCs, and contributes to the proliferation and maintenance of B cells. Additionally, Flt3L synergizes with other hematopoietic growth factors in the development and expansion of myeloid cells (e.g., with **SCF**, **IL-3**, **IL-6**, **GM-CSF**, and **G-CSF**) and promotes the expansion and maintenance of lymphoid progenitors in synergy with **IL-7**. Increases in Flt3L can massively expand migrating and resident DC populations in the periphery, and dysregulation of the Flt3L-Flt signaling axis has been identified as an important contributor to leukemia.

I-309 (CCL1)[36] – Ligand of the CCR8 receptor that may play a role in the recruitment of T cells and eosinophils to sites of inflammation, and has been shown to play an important role in the trafficking and activation of ILC2 cells to promote type 2 immunity. I-309 has also been shown to drive Treg recruitment[37]. Elevated I-309 levels are associated with allergic diseases such as asthma and with poor prognosis in cancer.

IL-1RA (IL-1 receptor antagonist)[3] Antagonist of the IL-1 receptor, IL-1RA blocks the effects of **IL-1α** and **IL-1β**. Thought to be an acute phase factor that balances the physiologic effects of IL-1. IL-1RA is often observed to be elevated in cytokine storm, and is likely induced as an negative regulator of IL-1-driven inflammation[38].

IL-6[39,40] **–** Plays a central role in innate immunity as a key factor driving acute phase protein release from hepatocytes, and in adaptive immunity by stimulating antibody release from B cells and promoting CD4+ T cell differentiation to Th2 (with **IL-4**) and Th17 (with TGF-β) subtypes. IL-6 is thought to be an important contributor to 'cytokine storm' (cytokine release syndrome, CRS) in conditions such as MAS, AOSD, and COVID-19. IL-6 blocking agents have been approved or are being investigated for the treatment of a wide range of autoimmune, acute and chronic inflammatory diseases including rheumatoid arthritis, Castleman's disease, juvenile idiopathic arthritis, cytokine release syndrome (including severe COVID19), among several others[41].

IL-8 (CXCL8)[42] – An important factor in the recruitment and activation of neutrophils, as well as monocytes, eosinophils, and mast cells via CXCR1 and CXCR2. As a CXCR2 ligand, IL-8 is also a pro-angiogenic chemokine that contributes to wound healing responses. Elevated levels of IL-8 are associated with chronic inflammatory conditions like psoriasis, rheumatoid arthritis, COPD, and asthma.

IL-10[43] – A potent anti-inflammatory factor. IL-10 promotes immunomodulatory Treg development, decreases inflammatory cytokine production and prevents the development of T cell-mediated immune responses. Despite its established role as an anti-inflammatory cytokine, IL-10 has been implicated as a potential contributing factor to the 'cytokine storm' in severe COVID-19[44].

IL-18[3,4] – Produced mainly by macrophages and DCs, IL-18 is a key factor in type 1 immunity. IL-18 acts synergistically with IL-12p70 to stimulate the expression of IFNγ in Th1 cells and ILC1s, sustains Th1 and cytotoxic T cell activation, and is a key component regulating ILC1 and NK cell function. Like IL-1β, IL-18 is activated following inflammasome formation. Some inflammatory and autoimmune diseases are thought to be mediated by dysregulation of IL-18 signaling, such as systemic lupus erythematosus, rheumatoid arthritis, type-1 diabetes, Crohn's disease, psoriasis, and graft versus host disease. IL-18 may also be an important contributor to 'cytokine storm' in conditions such as macrophage activation syndrome, adult-onset Still's disease, and COVID-19.

IL-27[45] **–** A pleiotropic cytokine with both pro- and anti-inflammatory functions. Contributes to Th1 cell differentiation and expansion at the onset of a type 1 immune response, but also inhibits the release of IL-2 and induces the release of IL-10, which suppress the Th1 response, so IL-27 may contribute to both the initiation and resolution of type 1 inflammatory responses. IL-27 has also been shown to inhibit Th2 and Th17-mediated immune responses and to promote the development of immunomodulatory Treg cells.

M-CSF (macrophage colony-stimulating factor)[17] **–** Hematopoietic growth factor that drives the replication, differentiation, activation, and survival of a variety of myeloid cells, most prominently macrophages with a possible bias to protective M2 polarization. Both protective and pathogenic roles for M-CSF have been observed in several inflammatory conditions, but it has been implicated in the pathogenesis of conditions including arthritis, pulmonary fibrosis, and atherosclerosis.

MCP-1 (Monocyte chemotactic protein 1; CCL2)[46] – Chemotaxis of CCR2-expressing immune cells, and regulation of T cells favouring polarization to the Th2 subtype. MCP-1 is also a pro-angiogenic chemokine

that contributes to wound healing responses. Elevated MCP-1 is associated with cardiovascular disease, allergic asthma, rheumatoid arthritis, and invasive cancer.

MCP-2 (Monocyte chemotactic protein 2; CCL8)[47] – Drives the recruitment of a variety of immune cells, including monocytes, eosinophils, basophils, DCs, NK cells, activated T cells, and neutrophils via CCR1, CCR2, CCR3, and CCR5.

MIG (Monokine induced by gamma interferon; CXCL9),
IP-10 (Interferon gamma-induced protein 10; CXCL10)[48,49] – CXCR3 ligands induced by IFNγ that drive the recruitment of macrophages, dendritic cells, NK cells, and Th1 cells to inflamed, infected, or neoplastic regions, and so are important chemokines that contribute to type 1 immune responses. MIG and IP-10 also contribute to the regulation of apoptosis and cell proliferation, and are angiostatic factors released late in wound healing responses that promote transition to the remodeling phase.

MIP-1β (Macrophage inflammatory protein-1β; CCL4)[47] – Regulates the trafficking of immune cells, including Th2 cells, B cells, monocytes, eosinophils, DCs, macrophages and NK cells, to sites of inflammation via the receptors CCR1, CCR4 and CCR5.

High levels of the analytes in this group may be associated with innate immune responses – IL-6 plays a central role in innate immunity as a key factor driving acute phase protein release[39,40], IL-18 is a pro-inflammatory alarmin released following inflammasome activation[3], and Flt-3L is an important factor in innate lymphoid cell development[50]. High results across this group may indicate severe systemic inflammatory responses such as 'cytokine storm' (cytokine release syndrome; CRS). IL-6, IL-10, IL-18, IL-8, MIG, IP-10, MIP-1ß, MCP-1, BCA-1 have been found to be important soluble mediators of cytokine storm[38]. The anti-inflammatory factors IL-1RA and IL-10 have been found to be upregulated in cytokine storm, likely representing an insufficient regulatory response. High values across the analytes in this group are frequently observed in conditions associated with CRS, such as macrophage activation syndrome (MAS), adult-onset Still's disease (AOSD), and systemic arthritis, as well as lymphoproliferative disorders such as hemophagocytic lymphohistiocytosis (HLH) and lymphocytic leukemia.

GROUP C – UNCLASSIFIED BIOMARKERS

RANTES (Regulated on activation, normal T cell expressed and secreted; CCL5)[51] – A chemokine contributing to both type 1 and type 2 immune responses, RANTES drives the recruitment of T cells (both Th1 and Th2), dendritic cells, eosinophils, NK cells, mast cells and basophils via CCR1, CCR3 and CCR5. RANTES is also a pro-angiogenic chemokine that contributes to wound healing responses. Release of RANTES is prominently associated with viral infections, and is also implicated in asthma, atherosclerosis, angiogenesis in cancer, and fibrotic diseases.

TRAIL (TNF-related apoptosis-inducing ligand)[52] - Apoptosis-inducing member of the TNF superfamily. TRAIL is an important factor in NK-mediated apoptosis and has potent anti-tumour activity by preferentially inducing apoptosis in cancer cells but not in normal cells. TRAIL has also been shown to promote the resolution of inflammation by accelerating apoptosis of neutrophils and has anti-inflammatory effects on T cell function by inhibiting the proliferation of Th1 cells, promoting Treg proliferation, and inducing apoptosis in autoreactive T cells and B cells.

Neither of these biomarkers were found to correlate strongly with any other in the panel.

GROUP D – TYPE 2/TYPE 3/MUCOSAL IMMUNE RESPONSE

Eotaxin-3 (CCL26)[53] – Recruitment eosinophils, basophils, mast cells, and activated Th2 cells, to sites of inflammation via CCR3. The eotaxins are major contributors to type 2 (Th2-mediated) immunity, and high levels are associated with allergic diseases such as asthma, atopic dermatitis, and allergic rhinitis, as well as parasite infections and other inflammatory conditions (i.e., eosinophilic esophagitis, gastroenteritis).

IL-16[54] – A ligand of the CD4 receptor, IL-16 acts as a chemoattractant and activating factor for CD4+ T cells, eosinophils, monocytes and DCs. IL-16 also potentiates IL-2- or IL-15-driven CD4+ T cell proliferation. Elevated secretion of IL-16 has been observed in asthma and inflammatory bowel disease.

IL-17F[8] – An effector cytokine of type 3 immune responses that is released by activated Th17 cells, IL-17F forms homodimers or heterodimers with IL-17A to promote host defense at mucosal barriers. Unlike IL-17A, IL-17F can also be produced by activated monocytes and epithelial cells. Elevated IL-17F (and dysregulated type 3 immunity) has been recognized as a key contributor to autoimmune disorders including psoriasis, rheumatoid arthritis, multiple sclerosis, as well as inflammatory bowel disease.

IL-20[55] – Pro-inflammatory cytokine that may facilitate the communication between leukocytes and epithelial cells, thereby enhancing innate defence mechanisms and tissue repair processes at epithelial surfaces[56]. IL-20 has been implicated in the pathogenesis of autoimmune conditions such as rheumatoid arthritis, psoriasis, and nephritis.

IL-21[57] – Pleiotropic cytokine produced by NK and T cells (most prominently Th17 and Tfh subsets) with broad effects in regulating both innate and adaptive immune responses, IL-21 is an important factor in promoting sustained immunity to chronic infections. IL-21 may also drive differentiation of naïve CD4+ T cells to Th17 cells (with TGF-β) and to Tfh cells (in the absence of TGF-β).

IL-23[58] **–** Drives the differentiation of Th17 cells (when present with IL-1) and induces IL-17 release. As a key regulator of the type 3 immune response, dysregulation of IL-23 contributes to autoimmunity, and elevated levels are associated with chronic bowel inflammation, inflammatory joint diseases, psoriasis, and multiple sclerosis. Th17 cells can acquire the ability to secrete IFN-γ/GM-CSF (the highly inflammatory and pathogenic Th17.1 phenotype) following prolonged exposure to elevated levels of IL-23 and IL-1β[59]. IL-23 blocking agents have been approved or are being investigated for the treatment of psoriasis, psoriatic arthritis, rheumatoid arthritis, Crohn's disease, and ankylosing spondylitis, and inflammatory bowel disease. IBD patients that do not respond to TNF blockers (indicative of a refractory phenotype) tend to respond well to anti-IL-23 antibody therapy[60].

IL-28A (IFNλ2)[61] – A type III interferon, IL-28A is released in response to viral infection and induces antiviral activity in target cells, and contributes to type 1 immune responses (the IFNλ receptor is restricted mainly to epithelial cells, in contrast to the wide distribution of expression of other IFN subtype receptors). There is evidence that IL-28A may also have protective effects in conditions associated with type 2 and type 3 inflammation[62].

IL-33[4,63] – A key cytokine in type 2 immunity, IL-33 induces type 2 cytokine release (IL-4, IL-5, IL-13) in Th2 cells, ILC2s, basophils, mast cells, and macrophages. Also, DCs activated by IL-33 promote Th2 polarization. IL-33 can also contribute to the resolution of inflammation by promoting tissue repair and integrity through the regulation of Tregs. IL-33 release is increased in mucosal tissues following tissue damage due to inflammation or infection and is thought to be a major contributor to allergic diseases such as asthma and atopic dermatitis, as well as chronic inflammatory or fibrotic diseases like ulcerative colitis and COPD.

LIF (Leukemia inhibitory factor)[64] – A pleiotropic cytokine with both pro- and anti-inflammatory functions, depending on the immunological context. LIF is expressed by Tregs and suppresses Th17 differentiation, and so may promote immune tolerance. LIF expression is induced by pro-inflammatory cytokines, and elevated levels of LIF have been observed in rheumatoid arthritis, airway infections, acute respiratory distress, asthma, sepsis, and neural inflammation, although its role is often thought to be anti-inflammatory and protective.

MCP-4 (Monocyte chemotactic protein 4; CCL13)[65] **–** Drives the recruitment of eosinophils, basophils, monocytes, macrophages, immature DCs, and T cells via CCR1, CCR2, CCR3, CCR5 and CCR11. Also induces eosinophil degranulation, basophil histamine release, and the release of pro-inflammatory cytokines from epithelial and endothelial cells. There is evidence that MCP-4 contributes to many diseases, most

prominently to airway diseases such as asthma and COPD, autoimmune diseases such as rheumatoid arthritis, and skin diseases such as atopic dermatitis.

SCF (Stem cell factor)[66] – Growth factor that contributes to homeostasis in a wide variety of tissues throughout the body, with a prominent role in the maintenance of early hematopoietic stem cells. SCF primes DCs to induce Th2 or Th17 differentiation and drives the maturation, degranulation, and homing of mast cells, and thus is implicated in the pathogenesis of allergic diseases such as asthma. SCF and its receptor c-Kit can also contribute to the development of several types of cancer.

TGF-α (Transforming growth factor α)[67] – A ligand of the EGF receptor, TGFα drives cell proliferation and stem cell survival, and contributes to developmental processes and tissue homeostasis throughout the body. Several immune cells, including neutrophils, monocytes, and eosinophils, express and release TGF-α upon degranulation, which may contribute to tissue healing following the inflammatory response. Persistently elevated TGF-α in the context of chronic inflammation may be a driver of fibrosis.

TPO (Thrombopoietin)[68] – Hormone that is the key driver in the generation of platelets, with deficiencies resulting in thrombocytopenia. TPO also contributes to the survival and expansion of hematopoietic stem cells and acts synergistically with IL-3 and SCF to enhance the development of a variety of immune cells.

TSLP (Thymic stromal lymphopoietin)[69] – Produced mainly by epithelial tissues in response to inflammatory stimuli, TSLP is a key regulator of type 2 immune responses. TSLP primes DCs to induce Th2 polarization, plays a role in the development of ILC2s, and drives the expression of type 2 cytokines (IL-4, IL-5, IL-13). Elevated levels of TSLP are associated with allergic diseases such as asthma, atopic dermatitis, and eosinophilic esophagitis.

> *High results of the analytes in this group could indicate a mixed type 2 (IL-33, TSLP, Eotaxin-3)/type 3 (IL-17F, IL-23, IL-21) immune response (additionally, SCF potentiates dendritic cells to induce either Th2 or Th17 differentiation, and IL-16 is a chemoattractant and activating factor for CD4+ T cells). IL-28A is associated with type 1 responses and may be a negative regulator of type 2 and type 3 responses, and LIF is released by Tregs and suppresses Th17 differentiation. Type 3 immunity is crucial in mucosal defence, and several analytes in this group are prominently derived from or act primarily upon epithelial cells, such as TSLP[69], IL-33[63], TGFα[70], IL-28A[61], and IL-20[56], which suggests that the group may reflect mucosal immune responses.*

GROUP E – EOSINOPHILIC INFLAMMATION

Eotaxin-1 (CCL11), -2 (CCL24)[53] – Recruitment of eosinophils, basophils, mast cells, and activatedTh2 cells, to sites of inflammation via CCR3. The eotaxins are major effectors of Th2-mediated inflammation, and high levels are associated with allergic diseases such as asthma, atopic dermatitis, and allergic rhinitis, as well as parasite infections and other inflammatory conditions (i.e., eosinophilic esophagitis, gastroenteritis).

> *High values of the analytes in this group could indicate a type 2 inflammatory response with eosinophil involvement[71].*

GROUP F – HEMATOPOIETIC GROWTH FACTORS

G-CSF (Granulocyte colony-stimulating factor)[72] – Hematopoietic growth factor that is an important driver of neutrophil development and proliferation. Also induces the mobilization of hematopoietic stem and progenitor cells from the bone marrow into circulation.

IL-3[73] – Originally known as multi-CSF, IL-3 drives the differentiation of bone marrow precursors to a wide variety of myeloid cells including monocytes, macrophages, neutrophils, eosinophils, basophils, and mast cells, and stimulates proliferation in hematopoietic stem cells. IL-3 also induces degranulation in mature basophils and mast cells and is an important factor promoting type 1 IFN release from plasmacytoid DCs

(pDCs). IL-3 acts on early hematopoietic precursor cells, and so can serve as a growth factor in the pathogenesis of acute and chronic myelogenous leukemia.

IL-7[74] – An essential factor in the proliferation, survival, and development of T and B cells. Also plays an important role in the development and function of ILC2s and ILC3s. Circulating levels of IL-7 increase in response to lymphopenia, and a deficiency in IL-7 signaling results in severe combined immunodeficiency (SCID).

IL-15[75] – Promotes adaptive immune responses by driving the proliferation and survival of activated T cells, along with the generation, activation and proliferation of ILC1s and NK cells. IL-15 may play a role in the development of hematological malignancies.

The analytes in this group are hematopoietic growth factors and could indicate the expansion and activation of lymphocytes (IL-7, IL-15) and/or leukocytes (G-CSF, IL-3).

GROUP G – HOMEOSTATIC CHEMOKINES

6CKine (CCL21)[76] **–** Drives the recruitment and trafficking of T cells via CCR7. Plays a key role in homing of lymphocytes to lymph nodes and Peyer's patches, and contributes to immune tolerance by regulating the steady state trafficking of DCs from mucosal tissues to lymph nodes.

CTACK (Cutaneous T cell-attracting chemokine; CCL27)[77] **–** Expressed primarily in keratinocytes in the skin, CTACK drives the recruitment and homeostatic trafficking of CCR10-expressing T cells to the skin. Elevated levels of CTACK have been observed in inflammatory conditions of the skin, including diffuse or limited cutaneous systemic sclerosis, atopic dermatitis, and psoriasis vulgaris.

MDC (Macrophage-derived chemokine; CCL22)[78] – Drives the recruitment of CCR4-expressing immune cells, most notably Th2 cells, to sites of inflammation. Higher levels of these chemokines are observed in allergic diseases such as asthma and atopic dermatitis, as well as in some cancers.

MIP-1δ (Macrophage inflammatory protein 1δ; CCL15)[79] **–** Drives the recruitment of immune cells, particularly eosinophils and basophils, via CCR1 and CCR3. MIP-1δ has been implicated in the pathogenesis of asthma, particularly in severe asthma with mixed Th1/Th2-driven inflammation and airway remodeling. MIP-1δ has also been observed to contribute to plaque instability in atherosclerosis, angiogenesis in lung cancer, and elevated levels are observed in patients with advanced sarcoidosis.

SDF-1α+β (Stromal cell-derived factor 1α+β; CXCL12)[80,81] – Ligand of the CXCR4 receptor with important roles in development, including the homing and maintenance of hematopoietic stem cells and the production of immune cells, including B cells, pDCs, and NK cells. May play a role in the onset or progression of cancer, viral infections, inflammatory bowel diseases, rheumatoid arthritis and osteoarthritis, asthma and acute lung injury, amyotrophic lateral sclerosis, and WHIM syndrome.

The analytes in this group are homeostatic (constitutively expressed) chemokines that can be upregulated in inflammatory contexts.

GROUP H – PLATELET ACTIVATION / WOUND HEALING

EGF (Epidermal growth factor)[82] **–** Important growth factor involved in development, homeostasis and wound repair in tissues throughout the body. It is also a major factor that drives the proliferation and survival of several cancers.

ENA-78 (Epithelial neutrophil-activating protein 78; CXCL5)[47] **–** Drives the recruitment of neutrophils, basophils and B cells via CXCR1 and CXCR2. ENA-78 is also a pro-angiogenic chemokine, and so can contribute both to wound healing and to the progression of some cancers.

GROα (CXCL1)[83] – Recruitment of granulocytes (most notably neutrophils), monocytes, and CXCR2-expressing CD8+ T cells and NK cells to sites of inflammation. GROα is a pro-angiogenic chemokine, and so can contribute both to wound healing and to the progression of some cancers.

PDGF AA, AB, BB (Platelet-derived growth factor)[84] – Growth factors comprised of homodimers or heterodimers of two PDGF subunits (A and B). PDGFs play a major role in development, and their release from activated platelets contributes to wound healing. They are important contributors to the development and progression of some cancers, as well as vascular diseases (i.e., atherosclerosis, pulmonary hypertension) and fibrotic diseases.

sCD40L (Soluble CD40 ligand)[85] – Soluble form of the costimulatory molecule in the TNF family that promotes immunoglobulin isotype switching and the development of humoral immune memory in B cells, and primes DCs to activate T cell responses. High sCD40L levels have been observed in several autoimmune diseases.

TARC (Thymus and activation regulated chemokine; CCL17)[78] – Recruitment of CCR4-expressing immune cells, most notably Th2 cells, to sites of inflammation. Higher levels of TARC are observed in allergic diseases such as asthma and atopic dermatitis (TARC has been shown to be upregulated following allergen challenge in atopic subjects), as well as in some cancers.

VEGF-A (Vascular endothelial growth factor)[86] – Growth factor that plays a major role in vasculogenesis and angiogenesis and is essential for vascular homeostasis. Tumour-secreted VEGF contributes to cancer progression by promoting angiogenesis in the tumour microenvironment. VEGF-A also contributes to retinopathy in several blinding eye diseases.

> *High levels of most or all of the analytes in this group could indicate a platelet activation/wound healing response, as all of these factors are stored in and released by platelets and/or take part in angiogenic, tissue remodeling or inflammatory processes during wound healing[87]. High results in this group are often observed in conditions typically associated with vascular injury, angiogenesis and/or thrombocytosis, such as AOSD, Kawasaki disease, juvenile arthritis, FMF, COVID-19 and Crohn's disease, whereas lower results have been observed in conditions associated with thrombocytopenia such as HLH, lymphocytic leukemia, and hematopoietic stem cell transplantation. Levels of the analytes in this group are significantly higher in serum samples than in plasma samples drawn from the same subjects.*

APPENDIX: CYTOKINE CLUSTERING ANALYSIS

Cytokine groupings were determined with unsupervised clustering analysis on >250 plasma-EDTA specimens provided to us for diagnostic testing from patients with a variety of inflammatory, autoimmune, and neoplastic conditions, detailed in our publication[88]:

> Polley DJ et al. Identification of novel clusters of co-expressing cytokines in a diagnostic cytokine multiplex test. *Frontiers in Immunology*. 2023-July-31 2023;14 doi:10.3389/fimmu.2023.1223817.

The groupings in this report were adapted to reflect functional similarities of certain analytes. Specifically, the placement of the analytes in groups E, F, and G were informed by common physiological functions of the analytes, and group C is comprised of the two analytes in the panel that did not correlate strongly with any other. The designations of physiological/pathological significance assigned to each grouping are speculative, based on an analysis of the immune signatures in our database of clinical specimens and on the functional/pathological roles of the analytes in each grouping established in the scientific literature.

ABBREVIATIONS:

Th – T helper lymphocyte

ILC – Innate lymphoid cell

DC – Dendritic cell

NK – Natural killer cell

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