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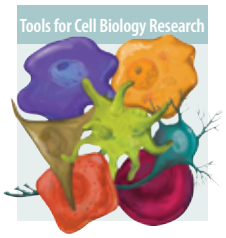
summer | 2007

cytokine BULLETIN

TOPICS IN CELL BIOLOGY

Inside



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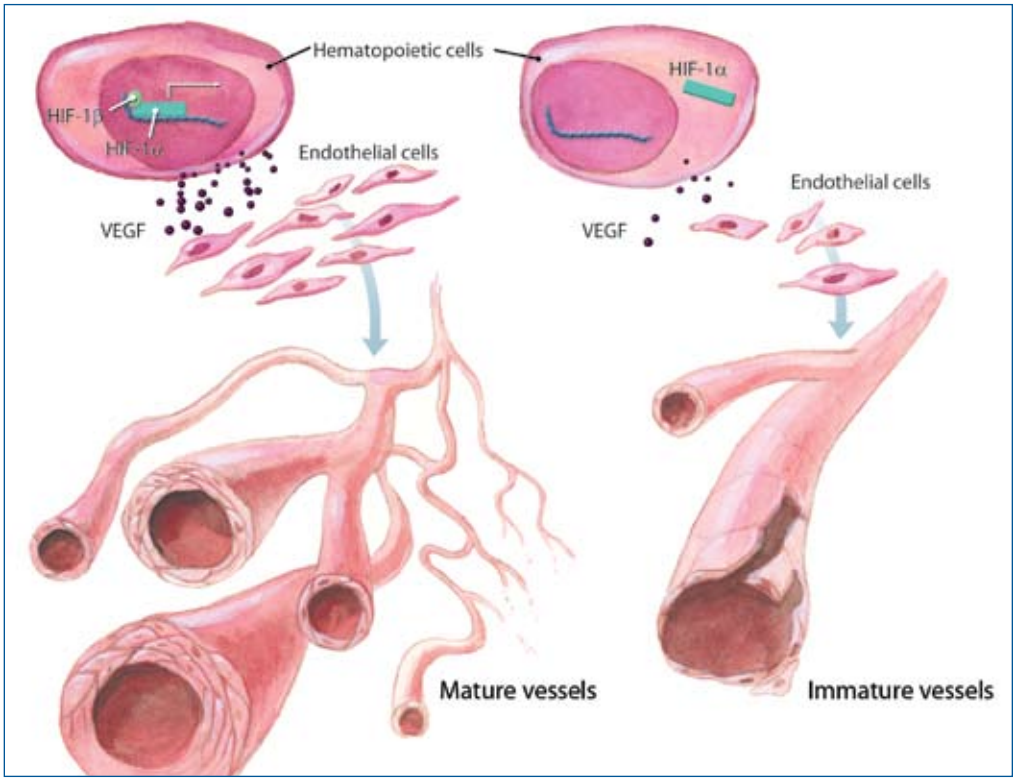

Figure 1. Differentiation of hematopoietic cells (HCs) and proper vessel development requires HIF-1-dependent factors. HIF-1 promotes the survival of HCs, which supply VEGF. VEGF induces vasculogenesis by increasing production and proliferation of endothelial cells. Loss of HIF-1β/ARNT results in HIF deficiency, increased apoptosis of HCs, reduction in VEGF production and endothelial cell number, and inadequate vessel growth and branching. (Adapted from reference 9).

HIF-1 & Vascular Development

Hypoxia is a condition defined by low oxygen levels (< 5%) and is a naturally occurring phenomenon during normal embryogenesis. Prior to the formation of a definitive embryonic vasculature and organ system, diffusion from extra-embryonic sites provides the oxygen necessary for development. During organogenesis, the local hypoxic environment seems to serve as a signal for blood vessel formation stimulating the production of angiogenic factors in a manner similar to the induction of angiogenic molecules that accompanies hypoxia in various tumor cells.[1] Hemangioblasts (primitive angiogenic cells) differentiate into endothelial cells that begin to form rudimentary tubes or vessels, and into hematopoietic stem cells that embed in the wall of these developing vessels.[2,3] This process (vasculogenesis) precedes the subsequent maturation and remodeling process of angiogenesis.[2] One molecule known to be essential for the initiation of vasculogenesis is vascular endothelial growth factor (VEGF). VEGF has pronounced mitogenic activity on vascular elements and is synthesized in response to hypoxia.[4]

HIF-1 (hypoxia inducible factor-1) is a key transcriptional regulator for hypoxic regulation of embryonic vascular development. HIF-1 is an oxygen-sensitive, dimeric complex composed of HIF-1α and HIF-1β/ARNT (aryl hydrocarbon receptor nuclear translocator) subunits.[5] During conditions of normoxia, HIF-1β is found in the nucleus, while HIF-1α is cytoplasmic and rapidly degraded by a ubiquitin-proteosome system. In mammalian cells, reduced oxygen levels permit the accumulation of HIF-1α protein in the cytoplasm. Subsequently, HIF-1α translocates to the nucleus, engages HIF-1β, and forms the HIF-1 complex that initiates VEGF transcription and mRNA stabilization.[6] Formation of the HIF-1 complex typically occurs during solid tumor formation (increased oxygen demand) and fetal development (inadequate oxygen delivery).

In the fetus, the HIF-1/VEGF connection is a current area of investigation to help explain the larger issue of circulatory system development. Mice lacking HIF-1 activity due to HIF-1α or HIF-1β/ARNT null mutations develop extensive cardiovascular defects including inadequate vessel formation and aberrant vascular remodeling.[7,8] The ubiquitous expression pattern and generalized placental

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Adiponectin & Apoptotic Cell Clearance

Adiponectin, also known as Acrp30, is an adipocyte-specific secreted protein with wide ranging effects on metabolism and inflammation. Adiponectin regulates glucose and fatty acid metabolism, insulin responsiveness, and adipocyte differentiation.[1] It has multiple anti-inflammatory properties (Box 1) that distinguish it from other adipocytokines such as leptin, resistin, and visfatin.[2] These various functions are mediated by the interaction of adiponectin with the receptors AdipoR1, AdipoR2, and, potentially, with cadherin-13/T-cadherin.[3,4] Adiponectin is the focus of much clinical interest for its involvement in the development of type II diabetes, obesity, and cardiovascular disease.[1]

Adiponectin consists of a globular domain and a collagen-like tail, both of which contribute to its association into multiple low, middle, and high molecular weight multimeric forms.[5] Adiponectin complexes are structurally similar to C1q and the collectins, molecules that participate in the clearance of apoptotic cells by macrophages.[6] The high circulating concentration of these molecules in the blood enables them to bind ligands with high avidity, suggesting regulatory functions.

Box 1

ANTI-INFLAMMATORY EFFECTS OF ADIPONECTIN[2]

- Decreased production of CXCL8, IFN-γ, IL-6, and TNF-α
- Increased production of IL-1ra and IL-10
- Decreased activation of NFκB
- Downregulation of vasular endothelial cell adhesion molecules
- Inhibition of macrophage-foam cell development

Inspired by the structural parallels between these molecules, Takemura *et al.*[7] recently elucidated how adiponectin also mediates the *in vivo* clearance of apoptotic cells, in a manner analagous to C1q and the collectins. In this mechanism, adiponectin preferentially binds to apoptotic blebs on dying cells, leading to phagocytosis by monocyte-derived macrophages. This adiponectin-dependent engulfment is restricted to early apoptotic cells, suggesting signal transduction via membrane receptors. Interestingly, this opsonization does not require AdipoR1, AdipoR2, or cadherin-13 on the target cells. Rather, the authors identify the apoptotic cell determinant as calreticulin.

Calreticulin, typically identified as a calcium-binding chaperone of the endoplasmic reticulum[8], has been shown to be upregulated in patches on the surface of apoptotic cells.[9] This calreticulin clustering is coordinated with a decrease in CD47, a molecule whose interaction with macrophage SIRP-α prevents phagocytosis of viable cells (Figure 1). In addition, calreticulin is expressed on the macrophage in association with the phagocytic receptor LRP/CD91.[5,9,10] The interaction of adiponectin with calreticulin on both the apoptotic cell and the macrophage is critical for the apoptotic cell clearance, as experimental knockdown of either calreticulin or LRP blocks adiponectin-dependent phagocytosis.[5] Apoptotic cells opsonized by C1q, or the collectins MBL, SP-A, and SP-D, are cleared by a similar mechanism involving calreticulin and LRP.[9,10] Such molecular redundancy is consistent with the partial decrease in apoptotic cell clearance observed in adiponectin knockout mice.[5]

The recognition and clearance of apoptotic cells helps prevent systemic inflammation.[12] These dying cells, if not cleared safely, would become necrotic, disintegrate, and release pro-inflammatory molecules. The failure to clear apoptotic cells has also been linked to the production of auto-antibodies.[12] Adiponectin-deficient mice, in fact, show increased severity of autoimmune symptoms.[5] Apoptotic cell clearance is a novel function of adiponectin that operates in tandem with adiponectin's other anti-inflammatory functions.

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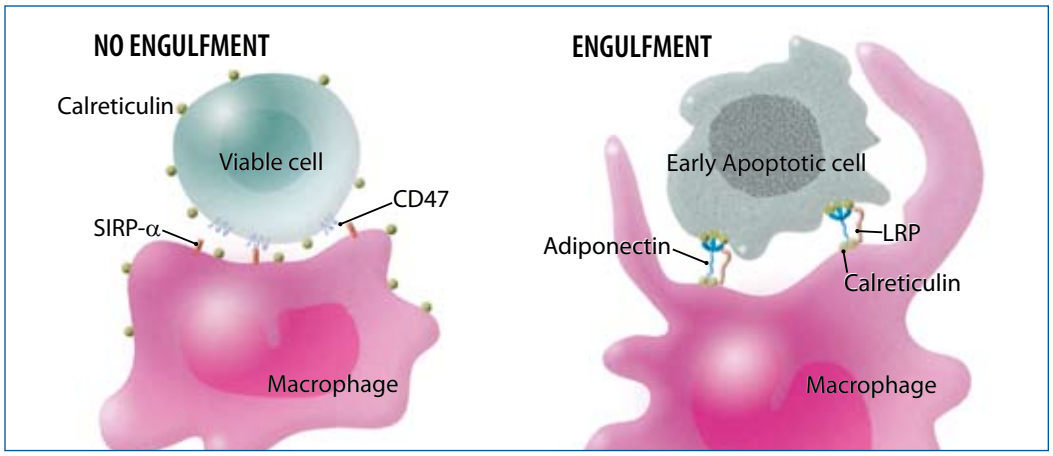

Figure 1. Viable cells express CD47 which binds SIRP-α on macrophages to prevent phagocytosis. Early apoptotic cells down-regulate CD47 and upregulate calreticulin in patches. Adiponectin recognizes calreticulin and promotes LRP-dependent clearance of apoptotic cells.

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Chemokines & Pregnancy

Establishment and maintenance of a proper maternal-fetal interface is essential for successful mammalian pregnancy. Not only must the non-pregnant uterus be transformed into a cellular and molecular environment capable of implantation and fetal survival (decidualization), but a maternally-based immune tolerance of the semi-allogenic fetus must also develop and be sustained. The trophoblast is the extraembryonic layer of cells that invade the maternal tissue and form the fetal portion of the placenta (chorion). Communication between fetal trophoblast cells and maternal immune cells dictates placental development and vasculogenesis during early pregnancy. It also establishes and maintains immune privilege throughout gestation. Recent studies identify chemokines as critical factors in this process.

Chemokines are a family of structurally-related, small, secreted proteins that render their chemoattractant effect through interaction with a subgroup of seven transmembrane domain G-protein-coupled receptors. Traditionally known to recruit immune cells to mediate inflammation, chemokines are now recognized as regulators in central nervous system development and hematopoiesis.[1,2] During placental development, cytotrophoblasts release CCL3/MIP-1α and decidual trophoblast cells lining maternal blood vessels secrete CXCL12/SDF-1, attracting $CCR5^+$ and $CXCR4^+$ Natural Killer (NK) cells, respectively, from the maternal circulation.[3,4] In response to local environmental cues (e.g. IL-15) NK cells recruited to the decidua (dNK cells) alter their phenotypic profile by down-regulating their chemokine receptors, and losing their cytolytic activity following engagement with HLA-G, a non-classical MHC Class I molecule expressed on trophoblast cells.[3,5,6] Recently, Hanna *et al.* proposed a positive role for dNK cells, demonstrating them to be sources of angiogenic growth factors following activation of the NK-activating receptor NKp44 by ligand-bearing cytotrophoblasts.[7] In addition to VEGF and PlGF, dNK cells release CXCL8/IL-8 and CXCL10/IP-10. These are chemokines that direct $CXCR1^+$ and $CXCR3^+$ trophoblast cells, towards endovascular invasion and vascular remodeling.[7] Thus, the mutual chemokine-mediated attraction between dNK cells and invading trophoblasts appears necessary for developing a functional maternal-fetal interface early in pregnancy.

The regulation of the inflammatory response by chemokines has also been implicated in miscarriage. A recent epidemiological study assessing chemokine levels in the serum of pregnant women found that elevated concentrations of CXCL5/ENA-78 and CCL5/RANTES were associated with higher risk of miscarriage.[8] Although both of these chemokines have been localized to decidual cells, it is uncertain if these circulating levels represent a placental imbalance.

Research by Martinez de la Torre and colleagues support a detrimental consequence theory of placental chemokines by reporting a beneficial role of the D6 chemokine receptor decoy in mouse fetal survival.[9] D6 is a silent receptor expressed on lymphatic endothelium that scavenges pro-inflammatory chemokines, thus reducing ligand availability to signaling receptors.[10] Following identification of D6 expression in human and mouse placenta, these authors investigated the effect of chemokines and D6 function in fetal immune privilege by subjecting pregnant wild-type (WT) and $D6^{-/-}$ mice to lipopolysaccharide (LPS) injection, an animal model of inflammation-related fetal loss. Systemic LPS injection induced an increase in both circulating and placental levels of inflammatory chemokines CCL22/MDC, CCL2/MCP-1/JE and CCL11/Eotaxin in WT mice, with exaggerated levels in $D6^{-/-}$ animals. Furthermore, LPS injection increased fetal loss, with significantly higher frequency observed in $D6^{-/-}$ mice relative to WT mice. Attenuation of LPS-induced fetal mortality was achieved by administering neutralizing antibodies to inflammatory cytokines. Collectively, the results support the critical role of inflammatory chemokines in pregnancy, and identifies D6's protective role in pregnancy as a scavenger receptor.[9]

Undoubtedly, future research will aim to unravel the impact of chemokines on fetal development, addressing issues such as time, location, relative concentration, and cellular environment. Such data will be critical in developing treatments for reproductive challenges, such as infertility, preeclampsia, chorioamnioitis, and miscarriage.

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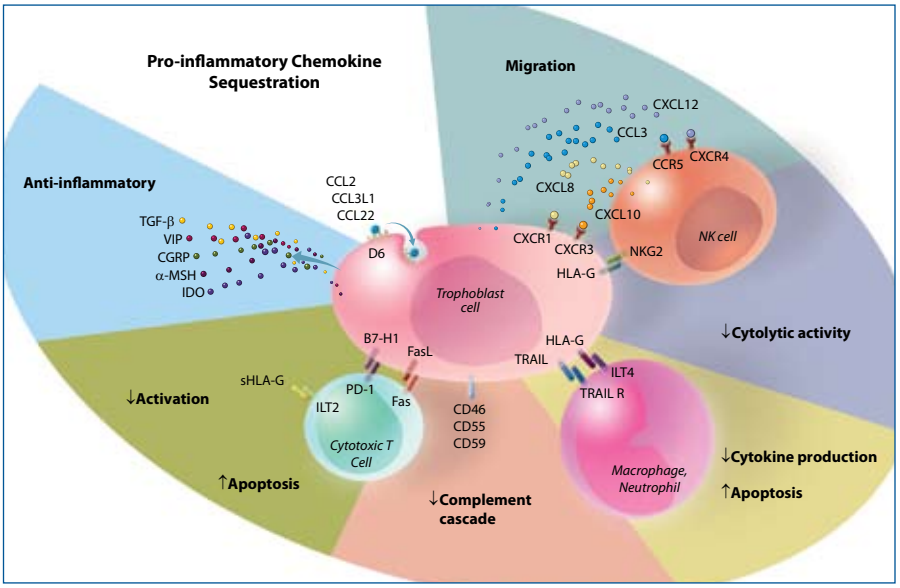

Figure 1. The trophoblast cell expresses numerous membrane receptors and soluble molecules that contribute to fetal immune privilege and promote placental development at the maternal-fetal interface.

Dkk-1: Upsetting the Balance in Rheumatoid Arthritis

Arthritis, defined as inflammation of the joints, occurs in many forms.[1] Although most arthritic conditions include joint remodeling, a distinguishing feature of rheumatoid arthritis (RA) is the presence of bone erosion without signs of repair. For example, bony growths called osteophytes are present in osteoarthritis (degenerative arthritis) and other forms of arthritis, yet absent in RA. Thus, RA includes both excessive bone resorption and inadequate bone replacement. Many proposed or recently initiated treatments for RA are aimed either at reducing inflammation or at curbing bone resorption, while less progress has been made toward enhancing bone replacement. A recent paper by Diarra and coworkers identifies suppression of signaling in the Wnt pathway as a key factor in the imbalance between bone resorption and replacement signals in RA joints.[3]

Signaling via the classical Wnt pathway is critical for bone deposition, both during development and bone remodeling in the adult.[3,4] This signaling is under tight control and is limited by multiple endogenous mechanisms. In one such regulation, Dickkopf proteins (Dkk) bind and promote the internalization of lipoprotein receptor-related protein (LRP)-5 or LRP-6. Blocking these Wnt receptor components effectively downregulates Wnt signaling. Diarra and coworkers have discovered that overexpression of Dkk-1 in RA joints leads to excess inhibition of classical Wnt signaling, and is a critical factor in the bone erosion seen in RA.[2]

The authors reported increased Dkk-1 expression in the serum or synovial fluid of three mouse models of RA, including one transgenic model that overexpresses the human inflammatory mediator TNF-α (hTNFtg). Dkk-1 expression was also increased in human RA, but was low in ankylosing spondylitis, a joint disease with a high prevalence of osteophytes. Treatment of hTNFtg mice with a Dkk-1 neutralizing antibody reversed the process of bone erosion but did not alter indicators of inflammation, suggesting that erosion is not a direct result of inflammation.

In addition to attenuation of bone loss, hTNFtg mice treated with Dkk-1 antibody exhibited osteophyte formation, suggesting an onset of bone deposition. Osteoblasts, the cells responsible for bone deposition, rely on Wnt signaling for their maturation.[4] Osteoblasts also control production of osteoclasts, the cells responsible for bone resorption. This occurs via production of the osteoclast-promoting growth factors M-CSF and RANKL/TRANCE, as well as the osteoclast-limiting RANKL/TRANCE antagonist, osteoprotegerin (OPG).[4,5] Wnt signaling enhances OPG production, while inhibiting RANKL/TRANCE. In the mouse arthritis model, inhibition of Dkk-1 allows increased production of OPG, increased population of osteoblasts, and decreased numbers of osteoclasts within the joint,[2] thus favoring bone replacement.

High Dkk-1 expression may be pathogenic and has been shown in other bone-reducing conditions such as multiple myeloma, Paget's disease and glucocorticoid-induced osteoporosis.[6-8] Several studies have proposed that Dkk-1 antagonism promotes bone deposition in these pathologies.[2,9] As TNF-α signaling is upstream of increased Dkk-1 production, therapeutic inhibition of TNF-α (an effective treatment of RA) is also likely to modulate Dkk-1 production.[1,2] In RA models, however, it has yet to be shown that a normal balance between resorption and deposition of bone can be re-established without incurring the production of osteophytes.

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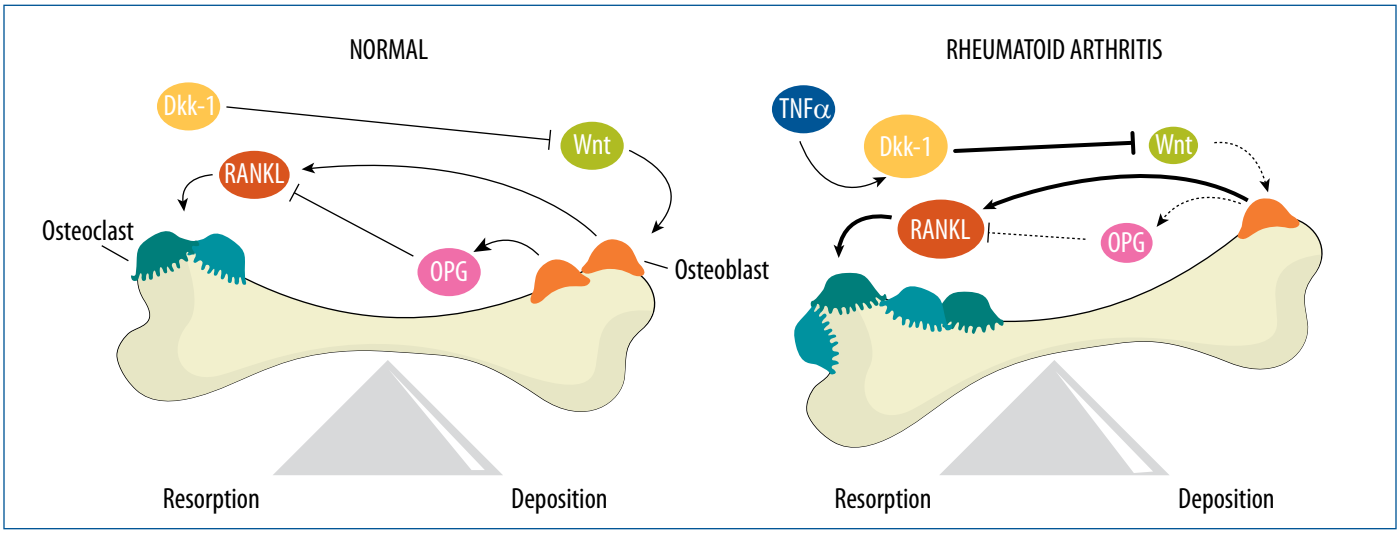

Figure 1. In normal joints, bone deposition by osteoblasts is in balance with bone resorption by osteoclasts. Wnt signaling is not over-inhibited by Dkk-1, and osteoclast formation is controlled by balanced amounts of RANKL/TRANCE and OPG. In rheumatoid arthritis, TNF-α promotes Dkk-1 overexpression, which in turn causes decreased OPG expression. These signaling events inhibit osteoblasts, while promoting osteoclasts and tipping the balance toward bone resorption.

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HIF-1 continued from page 1

function of HIF subunits during embryogenesis confound the analysis of vascular irregularities seen in HIF-deficient embryos. In order to evaluate the role of HIF-1 in vascular and hematopoietic development of the embryo independent of deleterious effects caused by abnormal circulation or placentation, para-aortic splanchnopleural (P-Sp) explant assays have been utilized.[9] These P-Sp explants are derived from a site within the embryo where definitive hematopoietic stem cells are committed from hemangioblasts, and the cultures support both angiogenesis and hematopoiesis.[10] As compared to those derived from wild type, P-Sp explants from HIF-1β/ARNT$^{-/-}$ mice exhibit reduced levels of VEGF protein, increased numbers of apoptotic hematopoietic cells, and abnormal vasculogenesis, angiogenesis, and hematopoiesis.[9] When sources for VEGF are added to the system, both endothelial and hematopoietic cell (HC) precursors appear.[9] Whether added exogenously or supplied via HCs, VEGF rescues endothelial cell production and vascular morphogenesis (see Figure 1). These results indicate that both decreased survival of, and VEGF production by, HCs contribute to the abnormal vessel development observed in HIF-1β/ARNT$^{-/-}$ explants, and suggest that HIF is critical for intraembryonic blood and vessel development.[9] These results may complement previous studies identifying HIF-1 signaling via the angiopoietin/Tie-2 system[11] and hypoxic regulation of VEGF receptor expression/activity on endothelial cells,[12] further elucidating the precise role of HIF-1 during vascular development.

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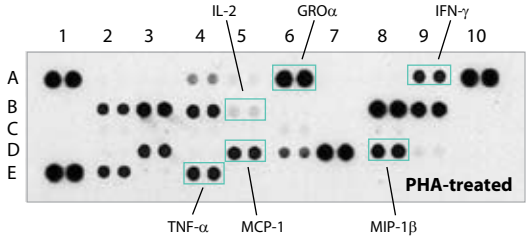

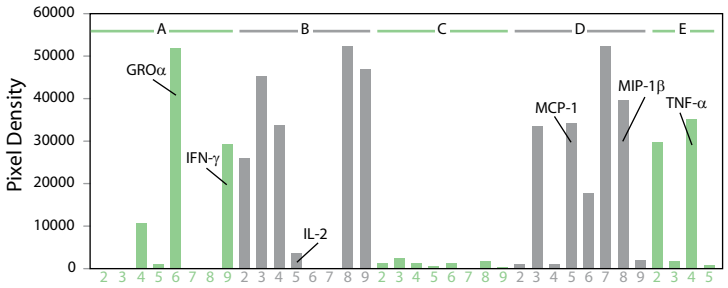

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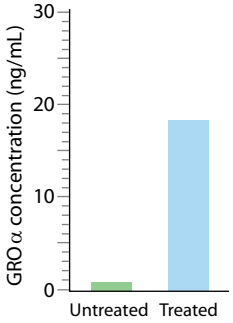
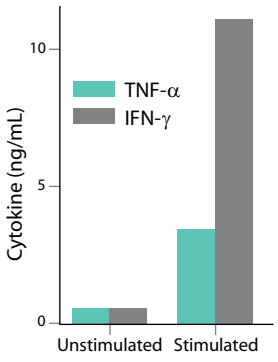
STEP 2

Quantify your results with our dependable ELISA/Assay Kits



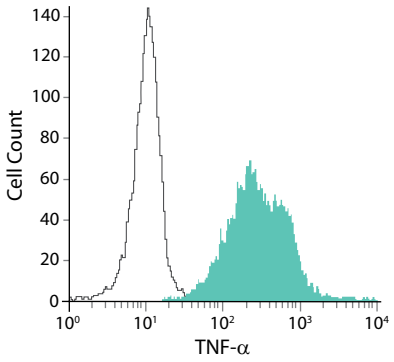
GROα in Peripheral Blood Mononuclear Cells (PBMC). PBMC were treated with PHA for 24 hours and assessed for CXCL1/GROα using the Human CXCL1/GROα Quantikine ELISA Kit (Catalog # DGR00).



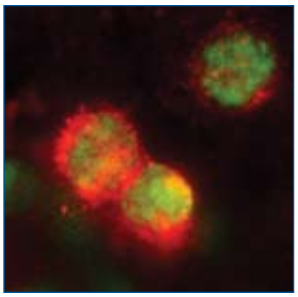
TNF-α and IFN-γ in Activated T Cells. The production of TNF-α and IFN-γ in the supernatant of PHA-activated human T cells (selected from peripheral blood mononuclear cells) are stimulated by CD3 activation using mouse anti-human CD3 monoclonal antibody (Catalog # MAB100). Cytokines were measured using human Quantikine ELISA kits for TNF-α (Catalog # DTA00C) and for IFN-γ (Catalog # DIF50).

STEP 3

Select from a wide range of antibodies and proteins to provide additional information in many types of experimental procedures



Intracellular Detection of TNF-α by Flow Cytometry. Human peripheral blood mononuclear cells treated with PMA and calcium ionomycin were stained with phycoerythrin (PE)-conjugated-mouse anti-human TNF-α monoclonal antibody (Catalog # IC210P; filled histogram). Staining with PE-conjugated isotype control (Catalog # IC002P; open histogram) highlights the specificity of the TNF-α antibody.



TNF-α Immunocytochemistry in Human Peripheral Blood Mononuclear Cells (PBMC). TNF-α was detected in human PBMC using anti-human TNF-α/TNFSF1A polyclonal antibody (Catalog # AF-210-NA). Cells were stained using a Rhodamine Red™ X-conjugated anti-goat IgG secondary antibody (red) and counterstained with FluoroNissl™ green.

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NEW TOOLS: Recombinant Active Protein Kinases

Protein kinases play critical roles in signal transduction, and their physiological activities in cellular processes are important in defining signaling pathways. Likewise, unregulated activities of protein kinases are of particular interest in understanding pathological processes such as cancer and chronic inflammatory diseases. Protein kinases often have multiple physiological substrates and cellular functions. Therefore, specific inhibitors of protein kinases are of interest in drug development. R&D Systems now offers a wide range of new recombinant active kinases that can be used in studies of their activities and inhibitors. These include members of the Src family, and kinases involved in the PI 3-kinase and MAP kinase signaling pathways.

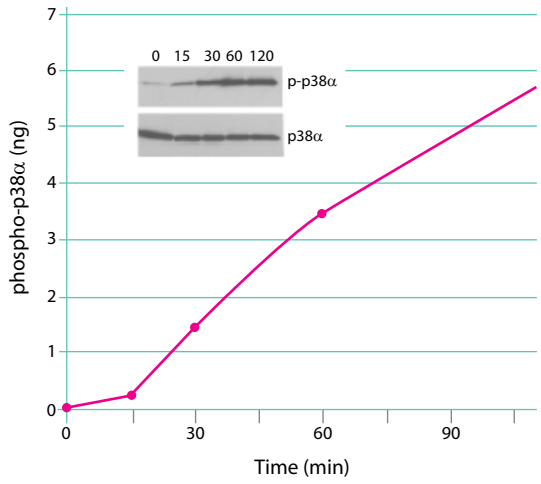

Figure 1. The phosphorylation of inactive p38α by recombinant human active MKK6 (Catalog # 1604-MK) was quantified at the indicated timepoints using the Phospho-p38α (T180/Y182) DuoSet IC Kinase Assay (Catalog # DYC869). The same samples were also immunoblotted (insert) with either anti-phospho-p38 MAPK polyclonal antibody (Catalog # AF869) or anti-p38α monoclonal antibody (Catalog # MAB869).

CATEGORY	ACTIVE KINASE	SPECIES	CATALOG #	ASSAY SUBSTRATE FOR ACTIVE KINASE
Cell Cycle	Chk1	Human	1630-KS	KKKVSRSGLYRSPSMPENLNRPR
	Chk2	Human	1358-KS	KKKVSRSGLYRSPSMPENLNRPR
	NEK2	Human	3706-KS	Myelin basic protein (MBP)
MAP Kinase	ASK1	Human	3575-KS	Myelin basic protein (MBP)
	ERK1	Human	1879-KS	Myelin basic protein (MBP)
	JNK1	Mouse	1776-KS	Recombinant Activating Transcription Factor (ATF)-2
	MAPKAPK2	Human	3705-KS	RRLNRQLSVA-amide
	MKK6	Human	1604-MK	p38 alpha (inactive; catalog # 869-P3), Myelin basic protein (MBP)
	p38 alpha	Human	1806-P3	Myelin basic protein (MBP)
	Raf-1	Human	3708-KS	Myelin basic protein (MBP)
PI 3-kinase	Akt1	Human	1775-KS	RPRAATF
	GSK-3 beta	Human	2506-KS	YRRAAVPPSPSLSRHSSPHQ(pS)EDEEE
	p70 S6 Kinase	Human	896-KS	CKRRRLASLR
	SGK1	Human	3200-KS	RPRAATF
Src	Lck	Human	3704-KS	Poly (Glu:Tyr, 4:1)
	Src	Viral	3389-KS	KVEKIGEGTYGVVYK
T cell Receptor	ZAP70	Human	3709-KS	Poly (Glu:Tyr, 4:1)