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Cytokines in Radiobiological Responses: A Review

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Abstract

Cytokines function in many roles that are highly relevant to radiation research. This review focuses on how cytokines are structurally organized, how they are induced by radiation, and how they orchestrate mesenchymal, epithelial and immune cell interactions in irradiated tissues.

Pro-inflammatory cytokines are the major components of immediate early gene programs and as such can be rapidly activated after tissue irradiation. They converge with the effects of ionizing radiation in that both generate free radicals including reactive oxygen and nitrogen species (ROS/RNS). "Self" molecules secreted or released from cells after irradiation feed the same paradigm by signaling for ROS and cytokine production. As a result, multilayered feedback control circuits can be generated that perpetuate the radiation tissue damage response. The pro-inflammatory phase persists until such times as perceived challenges to host integrity are eliminated. Antioxidant, anti-inflammatory cytokines then act to restore homeostasis. The balance between pro-inflammatory and anti-inflammatory forces may shift to and fro for a long time after radiation exposure, creating waves as the host tries to deal with persisting pathogenesis.

Individual cytokines function within socially interconnected groups to direct these integrated cellular responses. They hunt in packs and form complex cytokine networks that are nested within each other so as to form mutually reinforcing or antagonistic forces. This yin-yang balance appears to have redox as a fulcrum. Because of their social organization, cytokines appear to have a considerable degree of redundancy and it follows that an elevated level of a specific cytokine in a disease situation or after irradiation does not necessarily implicate it causally in pathogenesis. In spite of this, "driver" cytokines are emerging in pathogenic situations that can clearly be targeted for therapeutic benefit, including in radiation settings. Cytokines can greatly affect intrinsic cellular radiosensitivity, the incidence and type of radiation tissue complications, bystander effects, genomic instability and cancer. Minor and not so minor, polymorphisms in cytokine genes give considerable diversity within populations and are relevant to causation of disease. Therapeutic intervention is made difficult by such complexity; but the potential prize is great.

Introduction

It would be unwise to try to cover the field of cytokines in one short review. A book would hardly do it justice. Our aim is simple. It is to present some general aspects of cytokine biology that we feel are most relevant for radiation researchers. Even with this limited discussion we can only sketch outlines and present examples that inevitably prejudice content by selection. We will focus primarily on the major rationale for studying cytokines in radiation responses, namely that they are induced to orchestrate complex mesenchymal, epithelial, and immune interactions that influence tissue damage and restore integrity and homeostasis through promoting angiogenesis and tissue regeneration or replacement by

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fibrosis (Fig. 1). Other less well described constitutive roles in maintaining tissue homeostasis, and in development, will not be covered here. We ask to be excused for numerous omissions caused by space limitations.

A Brief History of Cytokines and their Characteristics

Cytokine biology grew out of studies in the late 1960s and 1970s that ascribed specific biological properties to soluble "factors" secreted into culture supernatants after cell stimulation. Such factors were often named after the cell type and the activity affected, for example lymphocyte activating factor, macrophage chemotactic factor and macrophage aggregation factor. This nomenclature primarily reflected the researcher's own field of interest, as in lymphokines (from lymphocytes), interleukins (inter-leukocyte communication), monokines (from mononuclear phagocytes), colony stimulating factors (CSFs for hematopoiesis), interferons (IFNs that interfered with viral replication), connective tissue "growth factors", and chemotactic chemokines. The chaos generated by such parochial and arbitrary categorizations dissembled a little when Cohen *et al.* introduced the term "cytokine" in 1974 (1) to counter the notion that only lymphocytes make "lymphokines" (2), but was only finally averted with the advent of molecular cloning. This showed some cytokines to be eponymous but, more importantly, that "cytokines" form a meaningful generic group of multiple structurally-related families with about 200 member proteins of around a few hundred amino acids that have a common purpose of regulating crosstalk between cells and tissues. By virtue of their crucial role in cellular communication, cytokines are prime targets for therapeutic intervention in many diseases including infections, chronic inflammatory and autoimmune conditions, wound healing and neoplasia. In the past, the complexity of cytokine networks often thwarted such efforts, but recently key driver cytokines are emerging in specific disease situations that can be targeted for therapeutic benefit (3).

Cytokine Characteristics

The broad characteristics of cytokines that led to their being considered as a generic group are summarized in Table I. They are highly potent molecules that are generally transiently expressed in response to stimuli. Responses are highly coordinated within a network of molecules that have interactive and overlapping functionality and whose profiles change with time to meet challenges. Cytokines extend their influence by choreographing alterations in cell adhesion molecules, immune recognition, cell death and survival, cell cycle arrest/proliferation, and metabolism so as to integrate cell and tissue responses. Our current cytokine networks have presumably been sculpted by major periodic epidemics. As a result, redundancy, strong genetic influences and extensive diversity are characteristics. This is in keeping with a critical role of ensuring the ultimate survival of the species by promoting flexibility of response between individuals. There is still much to be learned about how coordination is achieved, but it is clear that cytokines hunt in packs with overlapping and sometime antagonistic functionality to drive responses forward.

The nature of cytokine biology infers that if the level of a specific cytokine is elevated in a disease situation or following radiation, it does not necessarily indicate how networked cytokines are functioning or if that specific cytokine is causally involved in pathogenesis. Cytokine levels are generally not good "end points" for disease in themselves and have to be directly related to functional events, for example by targeted inhibition. A positive result may show the cytokine to be a major driver of the pack causing a condition, but a negative or equivocal result may simply indicate a high level of redundancy. An additional interpretative complication is that levels of mutually antagonistic cytokines may be elevated at the same time. The balance within each cytokine network and within each individual has

to be characterized if mechanisms are to be fully understood and if therapeutic intervention is to have any hope of success.

Finally, most of the information on the roles of cytokines in human health and disease comes from immunoassay of levels in the circulation. These are influenced by half-lives and rates of secretion and may not relate directly or causally to events within tissues. Analyses of cytokine localization in cells and tissues by mRNA or immunohistochemical analyses are generally performed in model systems and can be more informative, although some cytokines are most effective in performing certain functions when cell-bound and detailed knowledge of juxtacrine cell–cell interactions involving cytokines is sparse.

In cancer, cytokine pathways are often dysregulated. Causative mutations are sometimes found such as in receptor-tyrosine kinase fusion genes that cause chronic myeloid malignancies (8). But, more often than not, cytokine overexpression results from dysregulated downstream control elements that enhance tumor growth, survival, invasion, and escape from immune surveillance. The simplest analogy for cancer is as a wound that does not heal (9). By the time tumors become palpable they have gone through initial recognition and acute inflammatory stages and may have been immunoedited so as to escape these control mechanisms. As a result, infiltrates most often resemble those in healing wounds with a preponderance of regulatory cytokines and cells as in the second phase in Fig. 1. As in all chronic lesions, there may be attempts to re-activate pro-inflammatory responses that can occur in waves over time (10), as is often seen after irradiation (11).

Cytokine Nomenclature

To this day the nomenclature of cytokine families is archaic and confusing, often bearing little relationship to either structure or function. For example, transforming growth factor-alpha (TGF-α) and transforming growth factor-beta (TGF-β) were first defined by their ability to promote a reversible “transformed” phenotype in normal rat fibroblasts in soft agar, but are in fact unrelated peptides with very different biological properties. In fact, the major effect of TGF-β is to inhibit proliferation of many cell types and it is highly immunosuppressive. One might be equally excused for thinking that members of the interleukin families are related. In fact, they share remarkably little homology even within a family. Many are produced by, or act on, nonimmune as well as immune cells, while exclusion of some and the inclusion of others, such as the chemokine IL-8 in the interleukins, seems arbitrary. Logical order has been brought to certain cytokine groups by consideration of common structural traits or common receptor utilization, the best example being the chemokines and chemokine receptors (Table III). However, even brief examination of the enormous size of the cytokine system would lead one to the conclude that bringing order to such apparent chaos would be a Herculean challenge. Even stunning advances in genomic sequencing and annotation, crystallography and NMR have not solved the issues of structural relationships.

Inclusion of “growth factors” as cytokines is controversial, although no one would dispute that some, for example members of the TGF-β family, are bona fide cytokines and that many “cytokines” act primarily as growth factors, e.g., the colony stimulating factors (CSFs). Perhaps the most persuasive argument for calling some growth factors cytokines is that they interact within the same networks and use similar signaling pathways as cytokines. In this review, the main focus will be the classic cytokines.

Structural Aspects of Cytokines

One might expect clues as to how cytokines evolved such diversity and redundancy to be embedded in their structures. There is no universal agreement as to their categorization, but

Table II shows some of the proposed family divisions. This was a complete mystery until, in a seminal study, Bazan (12) noted that a large group of cytokines share a common protein fold, the four α-helix bundle. These cytokines could be subdivided into short- and long-chain forms based on the length of their core α helices (Table II). How this "cytokine" fold is preserved in the midst of the huge diversity displayed by the primary structures is still a mystery. Other structural features within the four α-helix bundle cytokines are sometimes used to provide finer categorization. The degree of consensus is not overwhelming, but the IFN and interleukin-10 (IL-10) families stand out as having similar yet distinct structures.

Outside the four α-helix bundle cytokines, IL-1 and IL-18 superfamily members are distinguished by shared β-trefoil structures formed by six two-stranded hairpins. The TNF superfamily shares β-sandwich trimeric structures. The fairly recently discovered IL-17 superfamily is distinguished by four highly conserved cysteine residues and the TGF-β superfamily is distinctive in having 9 cysteine residues, eight of which create a characteristic cysteine knot structure, while the ninth is involved in dimerization. The more than 50 chemokines from 4 distinct subfamilies based on the position of the conserved cysteine residue. They are involved primarily in the trafficking of different immune cells to sites in the body, in particular inflammation. The gradients that form by their localized secretion are important in directing such traffic. In general, CC chemokines steer polymorpho-nuclear leukocytes, monocytes, and NK cells while CXC chemokines focus more on directing B and T cells (13).

Cytokine Receptors and Signaling

Such is the diversity of cytokines that structural relationships are more easily seen in their receptors where essential signaling domains are highly conserved (Table II). A general principle is the need for receptor dimerization and aggregation for signaling, although this is often by itself insufficient and additional adaptor molecules are needed (14).

Both type I and 2 receptor families bind 4-helix bundle cytokines. Type I cytokine receptors recognize many interleukins and CSFs. They bind ligands through a shared conserved 200 amino acid cytokine homology domain (CHD) (12) and require a WSXWS motif for activation (15). Type II cytokine receptors are similar to type I receptors but lack the WSXWS motif. They bind IFNs, IL-10 homologs and IL-28-29 family members. Both type I and 2 receptors lack intrinsic tyrosine kinase activity and use Janus tyrosine kinases (Jak) and STAT proteins as intracellular signaling mediators.

A remarkable feature of the type I cytokine receptors is the potential for promiscuity and pathway interconnectivity that is brought about by sharing of signaling chains (II). In all, about 27 cytokines signal through 3 common chains (16). In addition, IL-12 and IL-23 cytokines have unique IL12Rβ2 or IL-23Rβ3 chains, but share an IL12Rβ1 signaling chain that has structural similarity to gp130 (17). There are many unanswered questions as to why this sharing evolved (18), but it is an efficient use of common signaling pathways while retaining ligand specificity. For example, there are more than twice as many chemokines as receptors; some receptors will bind more than one chemokine while some chemokines can bind several receptors. This functional pleiotropy and redundancy also indicates the degree of control exerted by spatial and temporal cytokine and receptor gene expression. In other words, "specificity" resides not solely at the level of ligand-receptor interaction, but by what cells express what and where (19). This organization reinforces the notion that cytokines form very tight, socially interconnected groups while retaining their own functional identities.

A remarkable example of controlled promiscuity is seen in IL-6 signaling. IL-6R is expressed in a limited fashion, primarily by macrophages, neutrophils, hepatocytes and

some T cells. “Classic” signaling requires association of IL-6R with gp130. However, IL-6Rs can be cleaved to an alternative soluble form, sIL6R; as can some other receptors, e.g., sIL-1R, sTNFR1, and sTNFR2. IL-6R is cleaved by disintegrin and metalloprotease ADAM17, which is activated by many signals, including IL-1, TNF-α and apoptosis (20). Interestingly, soluble IL-6/IL-6R complexes can “trans-signal” through gp130 and since gp130 dimers are ubiquitously expressed on all cells, the spectrum of IL-6 targets and the cytokine's functional impact is greatly expanded. Gp130 expression can be further enhanced, e.g., by IL-10, to increase cellular sensitivity to trans-signaling (21). Importantly, “classic” IL-6 signaling has regenerative and anti-inflammatory consequences, while trans-signaling is responsible for many of IL-6′s pro-inflammatory effects (20). Given the role of IL-6 in inflammation and cancer, targeting soluble complexes with soluble gp130 is being developed as a potential therapy (22) that may be relevant to RT, as sIL-6R has been shown to act with radiation-induced IL-6 to protect cells from radiation cytotoxicity (23); although IL-6 may affect radiation responses differently in different cancers.

IL-1R and IL-18R are prominent members of the immunoglobulin (Ig) superfamily that includes CSF-1R, PDGF-Rβ and stem cell factor receptor (c-Kit). IL-1 and IL-18 are structurally closely related cytokines whose receptors of which have signaling Toll/IL-1R (TIR) domains, also present in toll-like receptors (TLRs), indicating a common ancestry. Signaling in response to IL-1 requires IL-1RAP in addition to IL-1R binding, while so-called “decoy” receptors such as IL-1Ra have an inhibitory role.

Other distinct cytokine receptor families are the TNFR family, the TGF-βR family and the chemokine receptors. The TNFR superfamily has in excess of 27 members (often called TNFRSF1-27) that share partial homology in their extracellular cysteine-rich domains. TNFRs can be subdivided into whether or not they contain so-called cytoplasmic “death domains” (DD). The ten that do can recruit adaptor proteins, in particular Fas-associated protein with death domain (FADD) that bridges receptor activation to the caspase 8 cascade and apoptosis, a process that can be inhibited by FLICE-inhibitory protein that binds to FADD and caspase 8. Blocking the apoptotic pathway can result in programmed necrosis (necroptosis) that is regulated by activation of receptor-interacting serine/threonine protein kinase 1 (RIPK1) and RIPK3. Another outcome of TNF-α binding to TNFR1 is when dynamic endosome-associated complexes form containing TNFR-associated death domain (TRADD) and RIPK1 along with numerous other proteins, whose activities are regulated by ubiquitination, proteolysis and phosphorylation (24). Under such circumstances, MAPK and NF-κβ pathways are activated and generate downstream inhibitors of apoptosis (IAPs) such as survivin, IAP-1, IAP-2 and X-IAP; effector caspases are blocked, cell survival and inflammatory cytokines are produced. These signaling outcomes by death receptors vary depending on the TNFR and cells that are involved. In contrast, the TNFRs that do not have DDs can bind TNFR-associated factor (TRAF) interacting motifs (TIMs) to signal MAPKp38, extracellular signal related kinase (ERK) and phosphoinositide 3-kinase (PI3K) as well as NF-κβ and JNK. They generally function as regulators of the DD pathways, as do 5 decoy receptors.

Members of the TGF-β superfamily of receptors (types 1, 2, 3) have intracellular serine/threonine kinase domains and can form homo- or heterodimers. TGF-β signaling is through activation of SMADS. There are 8 SMADS belonging to 3 functional classes. Receptor-regulated SMADS are directly phosphorylated by type I TGF-βR through the intracellular kinase domain. These bind to a common mediator co-Smad4 to initiate gene transcription. Inhibitory iSMADS6 and iSMADS7 compete with SMAD4 to regulate transcription. The chemokine receptors are G-protein coupled with 7 transmembrane domains and their nomenclature reflects that of the chemokines (Table II).

The Early Response to "Danger"

Important for our understanding of radiobiological responses is how spatially and temporally integrated cytokine gene expression patterns unfold with time to direct tissue responses (Fig. 1). Regulation of gene expression is exerted transcriptionally and post-transcriptionally through a multilayer composite of genetic elements and processes, including DNA methylation, chromatin structure and remodeling, DNA sequence variants, RNA binding proteins, and micro-RNAs (miRNAs) so that rapid primary relatively promiscuous responses are mechanistically distinct from later more restricted responses. Immediate early gene responses are under the control of promoters that often have CpG islands. They involve constitutively active chromatin and are independent of nucleosomal remodeling complexes and contrast with later gene expression programs that may require gene demethylation and/or chromatin remodeling, with control being programmed into DNA structure at an early developmental stage and during lineage commitment (25).

Many inflammation-related cytokine genes (e.g., TNF-α, IL-1, IL-6, IL-8, IFNs, G-CSF, VEGF, and EGFR) fall into this category, being activated within minutes to hours after an exogenous signal without *de novo* protein synthesis. Control is exerted primarily by adenylate\uridylate (AU) elements in their 3′UTR regions (26, 27). Binding at such sites by cell-type-specific trans-acting binding proteins or microRNAs (28) causes immediate changes in mRNA abundance by transcript stabilization, although redox-sensitive proteins (29) and chromatin structure (30) also regulate expression. Importantly, polymorphisms and mutations within the 3′UTR have been associated with various diseases including radiation-induced cancer (31) and may contribute to inflammatory carcinogenesis. Other major groups of genes with AU rich elements are proto-oncogene transcription factors (e.g., c-jun, c-fos) or are involved in metabolism (e.g., GLUT1) or in redox regulation (iNOS, thioredoxin reductase, COX-2). Not surprisingly, radiation induces an immediate early gene response with rapidly increased expression of some proto-oncogenes and cytokines (32, 33). Since radiation-induced cytokines reappear much later (34), it is likely that these later responses are more cell-type restricted than those for initial "danger" responses. Examples of more restricted responses might be maturation of antigen presenting dendritic cells (DC) to present antigen, induction of regulatory T cells (iTregs) to terminate responses (35, 36) or radiation-induced senescence in keratinocytes (37). In the last example Bmi-1, a polycomb group protein, was shown to epigenetically silence NOX genes and mitigate radiation induced genotoxicity.

Radiation-induced rapid changes in redox-sensitive proteins could be important in many aspects of "danger" signaling, although this aspect of radiobiology is little studied. However, it is easy to see how, for example, cysteine oxidation may modulate the action of multiple redox-sensitive proteins (38) and one could imagine that there may be distinctive redox requirements for triggering of such molecules, which would be impacted by the basal redox status of the cell and the amount and type of ROS/RNS generated and their intracellular location. Importantly, the oxidative stress that follows irradiation can also result from the actions of pro-inflammatory cytokines.

Radiation-inducible redox-sensitive transcription factors include NF-κβ, early growth response factor (Egr1), and AP-1 (39) that are heavily involved in inflammatory cytokine production. Other ROS-responsive molecules of importance in radiobiology would include the protein mutated in ataxia-telangiectasia (ATM) (40), redox-sensitive phosphatases that may be responsible for rapid phosphorylation of EGFR and PDGFR (41) after irradiation and that can lead to further ROS generation (42), and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) that reacts to oxidative stress to activate the powerful mediator of cell survival and proliferation, Akt, and other kinases (43). Downstream of Akt, mTOR along with AMPK pathways that sense cellular nutrient and energy levels are also

receptive to redox changes, and can downregulate biosynthetic processes, including proteasome function (44), often results in further ROS production, autophagy and eventual cell death. The redox-sensitive transcription factor HIF-1, which is a major contributor to angiogenic cytokine production, is also activated by pro-inflammatory cytokines and is another example of their link to oxidative stress (45).

Cytokines and Radiation Converge in Free Radicals

Ionizing radiation effects converge with pro-inflammatory cytokines in that both generate free reactive oxygen and nitrogen species (ROS and RNS) such as superoxide, nitric oxide, hydroxyl radicals, peroxynitrite and their derived products (46). Some cytokines and growth factors, including those as diverse as TNF-α (47, 48) and EGFR (49), generate cellular ROS and actually require ROS for signal pathway activation. Conversely, anti-inflammatory cytokines, such as TGF-β, IL-10 and IL-4, tend to inhibit ROS/RNS-mediated effects and display anti-oxidative properties (50–52), although as always this may vary with the cell type and circumstances. This yin-yang feature is intrinsic to cytokine networks and suggests that redox is the fulcrum on which pro-inflammatory and anti-inflammatory responses are balanced (Fig. 2). This might explain why free radical scavengers, such as *N*-acetyl cysteine (NAC) (53), amifostine (54), or superoxide dismutase mimetics (55), can lower pro-inflammatory cytokine expression.

Questions arise as to the extent to which radiation responses and inflammatory cytokines mutually influence each other through ROS/RNS generation. Given the short half-life of ROS in cells, where, when, and how much of ROS is generated will impact their persistence and their abilities, for example, to deplete free radical scavengers, to change cellular redox status, to impact transcriptional signaling and to cause cell death. At clinical doses, radiation-induced ionizing events occur more or less randomly, unlike most oxidative stresses that act primarily at membranes. Classical radiobiology indicates that ROS generated per Gy through radiolysis of water will be short-lived and low in number in comparison with most oxidative stresses, at least for equivalent cell kill. The high cytotoxic efficacy of ionizing radiation is attributed largely to ROS generated within 2 nm of DNA and the formation of complex DNA DSBs, whereas the main sources of cellular ROS generated in response to oxidative stresses are generally mitochondria, membrane-bound nicotinamide adenine dinucleotide phosphate oxidases (NOX), or other oxidases. These may be secondarily linked to DNA damage response pathways (56–58). However, there is growing evidence that radiation also can generate ROS from these sources without nuclear intervention by damaging mitochondria, activating NOX or other oxidases (57, 58), or causing ATP release, ion channel activation (59) and purinergic signaling (60) (Fig. 3).

The consequences of secondary ROS production from pro-inflammatory cytokines after radiation exposure can be profound. High levels are likely to cause cell death and further perpetuate DNA damage, while lower levels may activate redox-sensitive signaling pathways such as those directed by nuclear factor-κβ- (NF-κβ) and mitogen-activated protein (MAP) kinase (61, 62). These pathways lead to the additional production of pro-inflammatory chemokines, such as IL-8 (CXCL8) and MIP-2 (CXCL2), and cytokines such as TNF-α and IL-1 both *in vitro* and *in vivo* (63–66). Interestingly, EGFR signaling can both induce inflammation and DNA damage through the generation of pro-inflammatory cytokines (67) and can also enhance DNA repair (68). Ultimately, cytokines may alter intrinsic radiosensitivity (69) by linking damage with cell fate decisions, including DNA repair, genomic instability, cell proliferation, differentiation, and death.

The radiation dose required to activate transcriptional responses by NF-κβ and pro-inflammatory pathways is of interest. Even low doses of radiation can be effective, for example in causing NF-κβ-mediated immune cell differentiation, although the optimal

activation dose tends to be in the region of 7–10 Gy (70), doses that are surprisingly also optimal for pro-inflammatory cytokine responses, at least in some studies (32). Radiation-induced bystander effects may be ascribed, in part, to these mechanisms (71).

DAMPS and their Receptors in Cytokine and ROS Production

The most obvious, though not the primordial, role of cytokines is to orchestrate mesenchymal, epithelial and immune cellular communications so as to restore homeostasis, as after radiation exposure. To fulfill these roles, cytokines network with endogenous and exogenous "danger" signals released from damaged tissues, as after irradiation. Tissue damage causes secretion or release of molecules that express damage-associated molecular patterns (DAMPS) into extracellular spaces that signal through conserved receptors that recognize broad features of molecules (Table III and Fig. 3). In this respect, DAMPS are similar to pathogen-associated molecular patterns (PAMPS) release during infection. Some of these DAMPS, like ATP, are secreted rapidly after irradiation and mediate cellular responses through activation of purinergic receptors that activate calcium channels (Fig. 3) (60). Others are secreted later or come from dead cells or the action of enzymes on the extracellular matrix. Mitochondrial peptides and DNA can act as DAMPS and if the damage is sufficient can cause systemic inflammatory response syndrome. The type of DAMP released with time may be important in defining the response that is made. Because the intracellular environment is generally a reducing one, molecules outside cells may be subjected to conformational change, making oxidation-specific moieties a particularly interesting source of DAMPS in response to radiation (72).

DAMPs and PAMPS signal through pattern recognition receptors (PRRs) (73). PRRs include the transmembrane TLRs and C-type lectin family receptors (Table III), endosomomal TLRs (TLR3, TLR7, TLR9), cytosolic retinoic acid-inducible gene-I-like helicases (RIGs) and receptors with nucleotide-binding domain (NOD) and leucine-rich repeats (NLR). Selectivity is broad but meaningful. For example, CpG-rich DNA from bacteria and viruses activate TLR9 in an endosomal compartment to generate type I IFN and other cytokines with antiviral properties. The link between receptors and cytokines is provided by adaptor molecules, such as myeloid differentiation primary response protein 88 (MyD88) and TIR domain-containing adaptor-inducing interferon-β (TRIF). These act through NF-κβ, MAP kinase, IRF and other down-stream signaling pathways so as to induce TNF-α, IL-1, IFNα/β, IL-10, and other cytokines (74). DAMPS therefore can initiate self-propelled cytokine cascades that primarily initially cause inflammatory tissue damage (11). PRRs at epithelial surfaces are equally important as those in immune cells in combating or facilitating entry of organisms into the body, including bacterial translocation from the gut after irradiation (75). In a radiation-damaged gut, various types of DAMPS may work in cohort with PAMPS to generate inflammatory infiltrates and activate innate immune defenses.

An example of a DAMP released after irradiation is the high-mobility group box 1 (HMGB1) protein, a chromatin-binding nuclear protein that signals through TLR4 to generate further ROS production (76) (Fig. 3). Through DAMPS, ROS and cytokine production multiple layers of self-amplifying feedback control circuits are created that prolong responses for long after the initial radiation-induced ionization events are completed (57). The consequences include vascular damage, interstitial fluid accumulation, inflammatory cell infiltration and creation of a lesion with a pro-oxidant microenvironment that is hostile to pathogens and cells alike and with a spatially and temporally expanded "danger" zone (77, 78) (Fig. 1). One consequence of this "danger" microenvironment is maturation of dendritic cells (DCs) that acquire the ability to present antigen so that adaptive immunity can develop. Other consequences may include certain radiation-induced "bystander" effects. Effects are likely to change spatially. At a distance from the lesion,

increased cell proliferation and survival may be promoted (79), which may be a mechanism to limit the spread of infection. One would also expect cytokine-mediated changes in cellular radioresistance (69).

Remarkably, some of PRRs form higher order oligomeric structures in the cytoplasm of some cells (74). In most cases, how and how often this process occurs is not well known. What is known is that NLR family members can assemble in response to appropriate "danger" signals to form inflammasomes (80). These can also be activated by microbial and host DNA independent of TLRs (81), where DNA binding proteins seem to play a major role. This mechanism may underlie systemic lupus disease.

The formation of the inflammasome autocatalytically activates caspase I (ICE) to cleave IL-1β and IL-18, which are "leaderless" pro-cytokines unable to exit the cell through normal secretory paths. The activation of IL-1β in this way may lead to cell death by "pyroptosis", which has characteristics of both necrosis and apoptosis. Pro-inflammatory NF-κβ driven synthesis of high levels of IL-1 in concert with an inflammasome secretory mechanism must send a strong "danger" signal to the body and could mediate many auto-inflammatory disease states (82). Recombinant IL-1R antagonist (Anakinra) or soluble IL-1R (Rilonacept) have been used to identify patients with such diseases and to distinguish them from those that respond better to anti-TNF therapies, such as infliximab (anti-TNF) or etanercept (TNFR2-IgG1 fusion product) (83). The role of inflammasomes in radiation-induced responses has yet to be defined but irradiated tissues often show a very strong IL-1β signal (84, 85) and this form of catalytic processing may be particularly important in the irradiated gut (86).

The involvement and impact of any cytokine will vary with the cell type/tissue and with time, but it is easy to see how ROS, pro-inflammatory cytokines, and DAMPS can mutually reinforce their relationship with time. Not surprisingly, after irradiation cells and tissues can express pro-inflammatory cytokines within minutes and re-expression can follow in waves for long thereafter (34). Survivors of Hiroshima (87) and Chernobyl (88) continue to have dysregulated cytokine expression. It also follows that many early and late manifestations of radiation damage can be cytokine-mediated. For example, early radiation-induced microvascular destruction after irradiation can be abrogated by an anti-TNF antibody (89), in keeping with the natural role of TNF-α in vascular effects that are manifest clinically in skin as erythema. TNF-α can also contribute to radiation-induced DNA damage, including γ-H2AX-staining double-strand breaks that may occur late after exposure and that often are associated with genomic instability (67). Protection against late radiation-induced demyelination in the brain is conferred by TNFR2 (90), suggesting a dichotomy between TNFR1 and TNFR2 pathways in mediating cell survival after irradiation. TNF-α and IL-1 therefore often appear as "driver" cytokines in inflammation.

Breaking the Free Radical-Cytokine Circuit

The fate of ROS in a cell may be short-lived but their effects are far-reaching and complex by virtue of their link to cytokines, cell signaling, and other interactive pathways. The production and activities of ROS need to be controlled, and this is generally achieved with high constitutive levels of free radical scavengers and antioxidants such as glutathione. Antioxidants are also generated in response to ROS that is induced by pro-inflammatory cytokines, radiation, and other oxidative stresses.

At the first level of protection, manganese superoxide dismutase (MnSOD/SOD2) can be generated in the mitochondrial matrix to control superoxide production in the site. Hydrogen peroxide results can require further degradation, for example, by catalase. Radiation can induce MnSOD expression through NF-κβ dependent pathways, as can cytokines and

microbial products, most notably IFN-γ and LPS and enhanced expression of SOD2 protects cells and tissues against radiation damage (91). NF-κβ therefore serves as a transcription factor for both pro- and antioxidant programs (92). Inducible nitric oxide synthase (iNOS) that generates nitric oxide (NO) can be produced by similar pathways after high-dose irradiation, or after low doses by a paracrine cytokine-dependent mechanism (93). NO can negate ROS, forming RNS with nitrosation and nitration. In rare cases, NO and RNS are able to promote apoptosis through inhibiting NF-κβ (94) but more often activate anti-apoptotic pathways (95). At the tissue level NO can directly effect blood flow and metabolism (96).

Although antioxidant enzymes involved in glutathione (GSH) synthesis can be generated rapidly through NF-κβ (97), this is generally part of a second level pathway that is controlled through another redox-sensitive transcription factor: NF-E-2-related factor-2 (Nrf2) (98). Nrf2 is normally bound in the cytoplasm by its redox-sensitive inhibitor Keap1. In response to oxidative stress, Nrf2 is released and binds the antioxidant response element (ARE) in the nucleus to transcribe numerous Phase II detoxification enzymes and antioxidant proteins (99). Under at least some conditions, radiation-induced Nrf2 activation does not kick in until several days after exposure, perhaps in response to late ROS generation and the depletion of antioxidant reservoirs (100). Nrf2 activation generally plays an important protective role in limiting the upregulation of NF-κβ activity and pro-inflammatory cytokine production and its depletion causes autoimmunity (102) and sensitivity to radiation (100).

There are many other examples of the importance of cross talk between redox-sensitive proteins and cytokine networks. For example, the antioxidant thioredoxin (Trx) in its reduced form binds and inhibits the MAP kinase kinase apoptosis signal-regulating kinase (ASK1). Oxidation of Trx-ASK1 by oxidants such as ROS drives the release and activation of ASK1 (103), leading to sustained activation of JNK, P38 and apoptosis (62). Alternatively, induction of Trx by cytokines such as IFN-γ, or oxidative or radiation stresses, blocks ASK1 activation and protects cells against apoptosis (104). Trx-ASK1 is therefore a molecular switch that converts a redox signal into kinase activation. This ASK1 pathway is required for pro-inflammatory cytokine production through TLR4 and p38 signaling pathways (105). Interestingly, the TNF-related adaptors TRAF2 and TRAF6 also form part of the ASK1 signalosome, linking the TNFR superfamily and TLR/IL-1R family to this ROS-responsive pathway (106).

Cytokines Hunt in Packs

The essential purpose of “danger” signaling is to alert the body so as to cause inflammatory host cell infiltration into the site. This varies in composition and function with time in a programmed manner. Initially, primarily neutrophils form a pathophysiological lesion to remove debris and pathogens if present. A balanced measure of cellular self-sacrifice by stressed local tissue cells and by the inflammatory cells [the “grateful dead” (73)] contributes and lymphocytes and macrophages follow. With time, cell proliferation and resistance to invasion and death in nonimmune “bystander” cells is enhanced, progenitor/stem cells are recruited from local stem cells by epithelial-mesenchymal cell transitions and from the bone marrow, and angiogenesis and vasculogenesis are stimulated. The transition from a pro-inflammatory, pro-oxidant environment to one that is more anti-inflammatory and antioxidant is critical for the tissue recovery processes. Later infiltrates of regulatory cells complete the regenerative process, or encourage replacement of damaged areas with fibrotic extracellular materials (Fig. 1). The ability of a tissue to recapitulate its original structure, which is present during prenatal life, is lost in adults, with some exceptions, and fibrosis and scarring driven by TGF-β is a common outcome (107). While fibrotic responses

may have the advantage of immediacy in maintaining tissue integrity, it comes at a cost of long-term loss of function and may inhibit the regenerative process.

Vascular damage after irradiation is a potentially important aspect of normal tissue and tumor responses that is compounded by a failure of angiogenesis, as can be demonstrated by the "tumor bed effect", where tumor growth in an irradiated site is slower than normal (108). In fact, hypoxia may be a general switch within any inflammatory site to drive factors like hypoxia inducing factor 1 (HIF-1) to reprogram a pro-oxidant, pro-inflammatory microenvironment to one supporting angiogenesis and wound healing through HIF-dependent cytokines, such as VEGF (Fig. 1). This has implications for irradiated tumors, where alternatively activated macrophages with an M2 phenotype accumulate under the influence of radiation-induced CSF-1 and stromal derived factor 1 (SDF-1) in areas of hypoxia that are generated by loss of microvasculature (109). Such macrophages produce large amounts of TGF-β and VEGF and can enhance tumor growth and wound healing. Hypoxia caused by irradiation of normal tissues may elicit similar consequences. The concept is that M1 pro-inflammatory macrophages "switch" into, or are replaced by, M2 macrophages with a change in the cytokine profiles (Figs. 1 and 2). The lack of angiogenesis and reliance on vasculogenesis in irradiated sites could lead to a vicious cycle of chronic activation of macrophages, fibroblasts and worsening hypoxia (110), more tissue damage and fibrosis; a nonhealing wound.

A moving picture emerges of diverse cell types interacting with a common purpose and with a high level of control being exerted over their functions and their existence. Control is in large part the purview of mutually antagonistic, cytokine-driven processes with pro-inflammatory, pro-oxidant pathways being opposed by, and giving way in time to, anti-inflammatory, antioxidant forces (Figs. 1 and 2). Loss of control has serious consequences, often ending in debilitating disease and even death. It is easy to see how snapshots, which do not take account of temporal aspects of responses, often paint cytokines as two-edged swords with roles in both pathogenesis of and recovery from disease.

Orchestration of these responses by cytokines requires considerable functional integration to drive them forward. To achieve such integration, cytokines are elaborated as functionally interactive cohorts that change in composition with time. These cohorts can be grouped in a very general way as: pro-inflammatory such as TNF-α, IL-1α and β, IL-17; angiogenic/vascular VEGF, TNF-α and FGF; anti-inflammatory IL-4, IL-10 and TGF-β; pro-fibrotic IL-6 and TGF-β; immune IL-2, IL-4 and IL7, and hematopoietic CSF1, GM-CSF, IL-3, EPO (Fig. 2). In fact, the cohorts should be viewed as interlocking, cross-talking networks that coordinate with other molecular and cellular systems to orchestrate tissue responses through changing redox, extracellular matrix, cell adhesion, cell cycle proliferation and cell migration to focus on the challenge at hand.

While cells of the immune system elaborate high levels of cytokines to effect host defense and maintain tissue integrity, this is not anarchy. Resident mesenchymal and epithelial cells are in the frontline of defense and they instruct immune cells how to behave in a site, in part through shared ligands and receptors and juxtacrine/paracrine interactions (75). Many, perhaps all, cell types share PRR recognition systems for DAMPS and PAMPS, though with differential expression.

Balancing Opposing Forces to Maintain Homeostasis

Moving beyond the acute phases of inflammation to later more directed responses, the most illuminating and dramatic example of coordinated expression and action of cytokines and division of labor comes from the discovery of distinct patterns of cytokines being produced by different antigen-specific helper/regulatory T cell subsets (Th/Tregs) (111). $CD4^+$ Th

cells recognize antigenic peptides 15–24 amino acids in length in association with MHC class II molecules on DCs through their T cell receptor-$CD3^+$ complexes. T cells must also receive a second verification signal through CD28 or a similar co-accessory molecule, or they will become anergic; a mechanism for maintaining peripheral tolerance to "self". DCs gain such molecules and other properties required for efficient antigen presentation by maturing in a "dangerous" microenvironment; a process that is switched off during healing. The potency of co-accessory stimulation was dramatically seen when volunteers were given an agonistic antibody to CD28 (TGN1412). They developed a cytokine storm and severe multi-organ damage (112).

Based on the signals received, $CD4^+$ naïve cells (Th_0) can differentiate along one of at least 4 pathways to form Th1, Th2, Th17 or iTregs, each with distinct cytokine profiles (Fig. 4). Antigen-specific responses in this way translate into broader effector mechanisms through cytokine secretion, affecting bystander immune cells and nonimmune cells that have the appropriate receptor profiles. For example, the M1/M2 profiles can be directed by the Th cell cytokines secreted and can feed back to either stimulate or inhibit lymphocyte responses. Th1 cells respond primarily to IL-12 to produce IFN-γ, GM-CSF and TNF-α, and cooperate with $CD8^+$ T cells and M1 macrophages to make cell-mediated responses that focus on elimination of intracellular viruses, bacteria and tumors, and that may also play a role in organ-specific autoimmune damage. Th2 cells, in contrast, are stimulated primarily by IL-4 to produce IL-4, IL-5, IL-6, IL-13 and IL-25. They assist B cells in the generation of antibodies that form allergic responses and are critical for expelling extracellular parasites and worms. Th17 cells differentiate in response to IL-6 or IL-22 to produce IL17, IL-21, IL-22, IL-23, and GM-CSF. Th17 cells have been implicated in the pathogenesis of many chronic inflammatory and autoimmune diseases (113) and they appear to be in a dynamic equilibrium with Tregs, as IL-6 can drive naive Tregs to become Th17 cells (114), a process that may be under HIF-1 control (115).

Tregs (116) are the other side of the immunological coin from Th cells. They are activated by antigen to maintain peripheral immunological tolerance and exert homeostatic control over inflammation. The presence of T cells that could suppress antigen-specific inflammatory T cell activity was recognized in 1971 by Gershon and Kondo, who called the phenomenon "infectious immunological tolerance" (117). The field fell into disrepute for many years, but re-emerged with the discovery of two subsets of natural and induced Tregs with mainly nonoverlapping antigenic repertoires that focus on controlling immune responses to "self" and on dampening inflammation. iTregs are induced by TGF-β and IL-2. They are distinct from the majority of Tregs that are naturally occurring thymus-derived nTregs. Although the respective roles of these subsets have yet to be fully elucidated, iTregs may be more important than nTregs in controlling inflammation at mucosal surfaces, while nTregs are more involved with tolerance to "self" (118).

Tregs display specificity through their T cell receptors but secrete anti-inflammatory and immunosuppressive effector cytokines, such as IL-10 and TGF-β, and collaborate with M2 macrophages to diametrically oppose Th1 and M1 cellular responses (Fig. 2). Another arrow in their quiver is their ability to convert pro-inflammatory extracellular ATP "danger" signals into immunosuppressive adenosine through induced expression of cell surface ectonucleotidases (Fig. 3), a process that is enhanced by radiation therapy (119), and in which HIF-1 and hypoxia might play a role (120). This is in keeping with the thesis that an antioxidant/adenosinergic microenvironment is generated that is tissue-protective which is the antithesis of pro-oxidant acute inflammation, and controls and neutralizes inflammation to promote healing. Recently, RT has been shown to increase Treg representation in mice and humans, perhaps to control radiation-induced inflammation (35, 121–125).

This dramatic T cell polarization leads to an important interpretation of disease progression that is based on the cross talk between Th subsets and their cytokines that form balanced opposing forces. The cytokine-driven switch from a pro- to antioxidant environment suggests that the fulcrum of this balance is redox (Fig. 2). In T cells, this polarization is orchestrated by the prevailing cellular microenvironment through a network of transcription factors: T-bet for Th1, GATA-3 for Th2, RORgammat for Th17 cells, and Foxp3 for Tregs (126). Thus, loss of the forkhead transcription factor Foxp3 results in Treg deficiency and multi-organ fulminating autoimmunity in humans and mice (127), while the IL-10 knockout mouse is an excellent model for chronic inflammatory disease (128).

The concept that distinct functional T cell subsets exist as balanced forces to maintain homeostasis within and outside the immune system has established validity. However, its extension to $CD8^+$ cells and "classically" activated M1 and "alternatively" activated M2 macrophages, with the former being pro-inflammatory and anti-microbial and the latter anti-inflammatory, wound healing and pro-angiogenesis (129) is less firmly established. DC1/DC2 subsets have also been proposed that selectively stimulate different Th subsets (130). Although they express distinct phenotypes and cytokine profiles, there is some controversy as to how "fixed" these subsets are and the degree to which they can be reprogramed. They may be more "plastic" than Th subsets that seem set in their lineages. Alternatively, even some T cell subsets show some evidence of plasticity as nTregs, but not iTregs, can be converted into Th17 cells by IL-6 with a distinct change in function (Fig. 4) (114).

In spite of these caveats, the concept of functional polarization of many cell types, whether transient or permanent and the cytokines they produce is critical for understanding many biological processes including the switches that drive progressive wound healing and the factors that establish the tumor microenvironment, with and without therapy. At any given time, what appear to be mutually antagonistic forces may be observed simultaneously. This is to be expected from a system that relies on the balance between opposing forces, expressed spatially and temporally, to maintain and restore control. As the battle ebbs and flows, one or the other aspect of events will be displayed, as is seen late after radiation exposure or in any other chronic condition. The tissue damage response to radiation will depend upon the same forces and redox responses.

"Radiation-Induced" Cytokine Gene Expression In Vitro and In Vivo

As has already been mentioned, exposure of cells and tissues to ionizing radiation *in vitro* and *in vivo* induces expression of many cytokines and growth factors. A few examples shown in Fig. 2 are: TNF-α, IL-1αα, IL-1β, (84, 131–133), type I IFN (134), GM-CSF (135, 136), IL-4, IL-5 (137, 138), IL-6 (136, 139), IL-10 (137), IL-12 and IL-18 (140), VEGF and bFGF (141), and TGF-β (142). Many appear as immediate early genes and legitimately qualify as "radiation-inducible". However, many confounders can alter the cytokine profiles produced after radiation exposure. Other factors will influence the late transcriptional, developmental and lineage-specific hierarchies that respond to tissue damage. The genetic make-up of the host, the influence of microbial products, tumors and other "extraneous" stimuli are possible influences. This begs the question as to what is meant by "radiation-induced". Several general points are worthy of consideration.

Obviously, radiation dose is important. One does not expect to see a straight linear dose-dependency for cytokine production. As mentioned previously, NF-κβ and pro-inflammatory responses generally require moderate doses of around 7–10 Gy to be optimal (11), but low-dose effects have also been observed (143). The strength of the signal and its persistence (e.g., dose fractionation/low-dose rate) would be expected to strongly influence the outcome. Indeed, one of the rationales for developing the standard 2 Gy fractionation protocol may have been to minimize the levels of pro-inflammatory cytokine expressed over

a short time period. Unfortunately, highly detailed analyses over wide dose ranges and times in multiple systems are difficult to perform and complete dose-response datasets are lacking.

It is important to realize that cytokine expression profiles change if cells are cultured *in vitro*, and are different from what is observed *in vivo* (144). Serum factors and even adherence to plastic can activate cytokine expression by macrophages (145). Furthermore, after whole-body irradiation the cytokines observed might be in response to microbes that have translocated across the gut or invaded the host due to radiation-induced immune suppression rather than being genuinely "radiation-induced". Microbial PAMPS, such as LPS, are far stronger pro-inflammatory stimuli than are radiation-induced DAMPS.

In vivo, the pathogenesis of radiation damage has a clear genetic element. The genetic bias in cytokine profiles demonstrated by different mouse strains in models of parasitic and autoimmune disease is well known. As a result, BALB/c and DBA/2 mice are often designated as Th2-oriented strains, and C57Bl/6 and C3H are Th1 strains. There is no reason to believe that this does not influence the response to radiation. This may be why Th2-type cytokine mRNAs, such as IL-4, IL-5 and IL-10, in addition to pro-inflammatory cytokines, were found to be increased after 5 Gy irradiation of Balb/c splenocytes (137). This simple Th1/2 designation is insufficient to describe all responses. For example, C3H mice develop potentially lethal pneumonitis following thoracic radiation, while C57Bl/6 mice resist this outcome and instead develop fibrosis (146). Both are "Th1" strains but distinct gene loci are involved (147). Both strains develop inflammatory infiltrates but in C3H mice pulmonary $Mac1^+$ macrophages increase dramatically as does pro-inflammatory cytokine production just before death (Fig. 5). C57Bl/6 mice control this macrophage-related cytokine response only to later develop IL-6/TGF-β associated fibrosis.

These "waves" of responses are seen in several different tissues and strains following radiation exposure (34). It appears most likely that the tendency to develop radiation-induced pneumonitis or lung fibrosis is determined by nonimmune host genetics, not by the genetics of the immune cells. The crossbred C57Bl/6 × C3H strain develops fibrosis, not pneumonitis, even if they have undergone a bone marrow transplant to give them a C3H immunohematopoietic system (McBride, unpublished data). A parallel for the interaction between immune and nonimmune cell types can be found in the way that tumors dictate the nature of their host cell infiltrates and modify systemic immunity through the release of cytokines and other modulators (148). Given these responses, it is not unreasonable to consider radiation-induced late effects as forms of chronic inflammatory responses that fluctuate in severity over time, much the same as in rheumatoid arthritis. Immune cells, including lymphocytes, are an integral feature of many radiation late effects. Their role remains rather a mystery but we know that thymectomy reduces radiation-induced pneumonitis and fibrosis in mice, suggesting that there is an autoimmune T cell component and that immune homeostasis is dysregulated (149).

Finally, it should also be remembered that all mouse strains have genetic features that might affect the cytokines they produce. C57Bl/6 have defects in phospholipase A II that is responsible for arachidonic acid release leading to production of eicosanoids (150). C57Bl/6 and DBA/2 have mutations in the P2X7R that governs responses to extracellular ATP (151). C3H/HeJ mice have a natural mutation in TLR4 that limits TNF-α production and LPS fails to protect them against radiation hematopoietic failure (152). Humans will express similar diversity.

Cytokine-Driven Responses in Irradiated Tissues

Radiation damage to many tissues ultimately culminates in fibrosis. Macrophages and other cells that accumulate in the damaged tissue that may previously have been pro-

inflammatory, switch to elaborating pro-fibrogenic cytokines like PDGF and TGF-β. TGF-β, which initially dampens macrophage and lymphocyte activation, begins to drives senescence of progenitor fibroblasts to fibrocytes, with consequent collagen synthesis and deposition (153–155). Thus, radiation-induced fibrotic remodeling of tissues represents a multi-cellular process, with initiation and sustenance of the fibrotic cascade by many different cell types.

In the central nervous system, cytokines and growth factors such as IL-1, FGF, PDGF, ciliary neurotrophic factor (CNF), NGF, and TGF-β appear to have a major role in regulating normal development and homeostasis (156). In addition, pro-inflammatory cytokines, especially IL-1 and TNF-α, have been implicated in the pathogenesis of CNS injury in multiple studies, including after irradiation (157, 158). In the brain, these pro-inflammatory cytokines also act as neuromodulators of sleep, neuroendocrine secretion and other functions (159).

The early effects of brain irradiation are due to damage to the cerebral microvasculature, leading to increased vascular permeability and loss of integrity of the blood-brain barrier (160). Edema and immune cell infiltration often lead to clinically significant nausea, vomiting and headaches, occurring in the acute (the first 24 h) and sub-acute (weeks to months) stages. Pro-inflammatory cytokines play a central role in all of these effects (84, 131). For example, micro-vascular changes occur in both irradiated and non-irradiated hemispheres as early as 3 days after irradiation that were abrogated when the mice were treated with anti-TNF-α monoclonal antibody prior to irradiation (89).

Late effects in the brain occur from six months to several years after radiation treatment. The resultant damage to the white matter can be very severe and interfere with the patient's quality of life. Chiang *et al.* showed increased TNF-α and IL-1 expression 2 weeks, 2–3 months and 5–6 months after mouse brain irradiation. This correlated with subacute and late loss of oligodendrocytes and demyelination (161). Loss of neural precursor cells residing in the subventricular zone and hippocampal dentate gyrus also occurs and may be implicated in somnolence and the inability to learn new tasks (131). Pro-inflammatory cytokines also drive the gliotic response to radiation (162–164). Loss of TNFR2 exacerbates radiation-induced brain demyelination (131), indicating the dual nature of the roles of TNF-α in the brain depending on receptor expression. Kim *et al.* also reported elevated levels of TNF-α weeks and months after unilateral mouse brain irradiation and TGF-β production (165).

In the lung, type II pneumocytes have been considered the traditional targets of irradiation. Electron microscopy reveals large-scale ultrastructural changes in the endoplasmic reticulum, mitochondria and plasma membranes of these cells, as well as in endothelium and type I pneumocytes. This leads to inflammation, desquamation of epithelial cells from the alveolar surfaces, edema, exudation into the alveolar spaces, thickening of the alveolar septa and alteration of the capillaries. An initial decrease in cell numbers is followed by an influx of neutrophils and lymphocytes into the alveoli (166, 167), although cells obtained by brochoalveolar lavage (BAL) do not have the same cytokine profile as do interstitial cells and are relatively inert (168). The final outcome is often genetically determined (Fig. 5), as noted above, with pneumonitis being associated with high levels of pro-inflammatory cytokines and fibrosis with IL-6 and TGF-β production (169). It has been suggested that in rats there is a switch to CD4 Th2 phenotype cells with TGF-β, IL-4, IL-10, and PDGF production that leads to activation of fibroblasts and increased collagen production, although this may be strain-specific (170). Antibodies to ICAM-1 and TGF-β, or transfer of soluble TGF-β type II receptor can block radiation pneumonitis (171–173). However, clinically this approach has yet to be shown effective.

In the rat intestine, within hours after irradiation, the ileal muscularis layer expresses high levels of IL-1β, TNF-α and IL-6 (174). The epithelium may regenerate normally but ulceration leads to accumulation of TGF-β and membrane thickening (175). Intestinal mesenchymal cells, mainly smooth muscle and subepithelial myofibroblast cells, are released from quiescence to begin the wound healing process and chronic fibrosis results with TGF-β driving the process. Radiation enteritis can be extensive, resulting in dysfunction, dysmotility, fibrotic structures with obstruction, fistula formation or bleeding, up to 8–12 months after irradiation. Injection of lipopolysaccharide, with induction of IL-1 in mice, prior to abdominal irradiation greatly increased peritoneal adhesions 2–4 months afterward suggesting a role for inflammation in this process (176).

In skin, a transient erythema is seen soon after irradiation. IL-1, IL-6, and TNF-α produced by activated dermal macrophages and Langerhans cells mediate proliferation and activation of keratinocytes, with early desquamation and late hyperkeratosis, and proliferation of fibroblasts, and the resultant fibrosis (177). Again, TGF-β stimulates fibroblast production of collagen in dermal layers (178, 179). Fetal wounds heal with minimal scarring: there is no acute response, and dermal fibroblasts are rare. TGF-β levels are low in fetal wounds as compared to adults, and injecting TGF-β into fetal wounds causes scarring, while injection of neutralizing antibodies to TGF-β-healed adult wounds causes minimal scarring (180). The suggestion is that this cytokine may initially be immunoprotective, dampening down the inflammatory response of macrophages and lymphocytes to radiation damage, but it negatively modulates regeneration by stimulating proliferation and activation of fibroblast collagen synthesis and deposition later on.

Conclusions

In spite of their complexity, links between cytokine and cytokine receptor structures and function are obvious with multiple family members overlapping to create diverse, socially interconnected networks that impact multiple aspects of radiobiology. While it is not easy to causally link increased cytokine levels to pathogenesis, the use of specific inhibitors has shown that in certain disease situations “driver” cytokines are critically important players and form useful targets for intervention. As a result, the number of clinically useful cytokine inhibitors has grown in recent years. Since the evidence that cytokines are intimately involved in radiation responses at all levels is irrefutable, manipulation of cytokine pathways is likely to be important in future radiation research and therapy.

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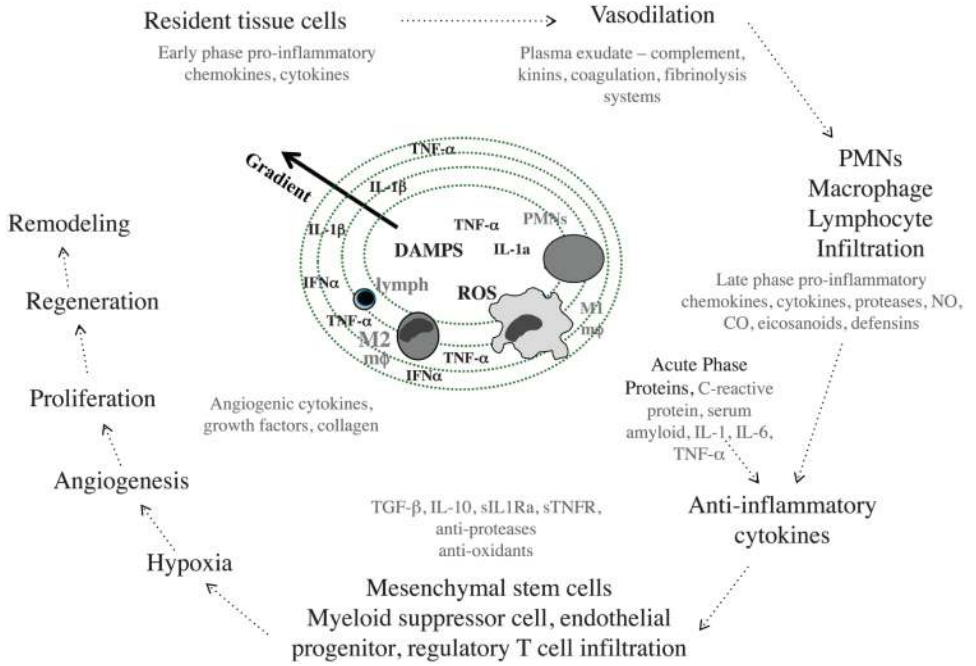

Fig. 1.

Cytokines drive the formation of inflammatory lesions working together with DAMPS to generate a pro-inflammatory, pro-oxidant microenvironment. The vasculature becomes leaky, allowing infiltration by neutrophils, and then macrophages and lymphocytes that migrate along chemokine gradients. Acute phase proteins, including cytokines, are generated along with a fair measure of cell death. In the periphery, cells may become more resistant to death and infection. Hypoxia may occur and in time the lesion resolves under the influence of anti-inflammatory cytokines and cells. Macrophages develop an M2 rather than an M1 phenotype. Angiogenesis and/or vasculogenesis assists either tissue regeneration or replacement with extracellular materials (fibrosis).

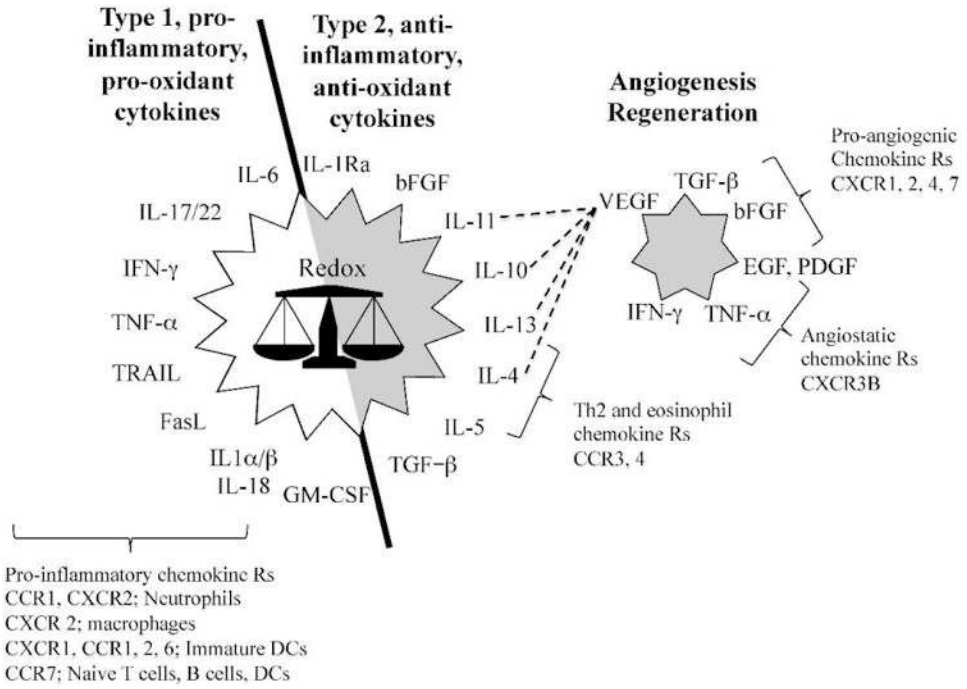

Fig. 2.

The yin-yang of cytokines. The balance between pro-inflammatory cytokines and anti-inflammatory cytokines is critical in determining outcome. Chemokines have preferred partners that link cell trafficking to function, as indicated. Angiogenesis, tissue replacement (fibrosis) and regeneration predominantly fall within the influence of the more anti-inflammatory axis.

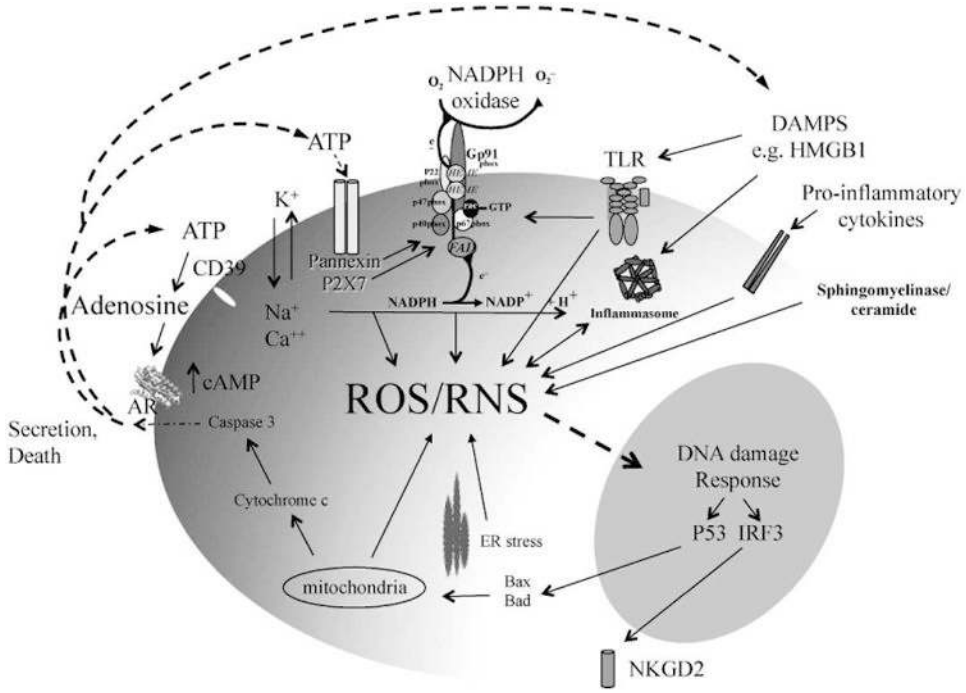

Fig. 3.

ROS can be generated from many sources following irradiation. Released nucleotides including ATP can activate P2X purinergic receptors to open the cation pore and trigger calcium-dependent intracellular processes. This is required for activation of NADPH oxidases that can also be activated by TLR signaling to generate superoxide. Radiation damage to mitochondria is another potential source of ROS. Further DAMP and pro-inflammatory cytokines signaling, the DNA damage response through Bax, and the formation of inflammasomes can all perpetuate ROS generation by forming positive feedback circuits. Adenosine can be generated from nucleotides by ectonucleotidases such as CD39 to signal through the adenosine receptors (AR) to negatively regulate inflammation, as does the production of anti-inflammatory cytokines.

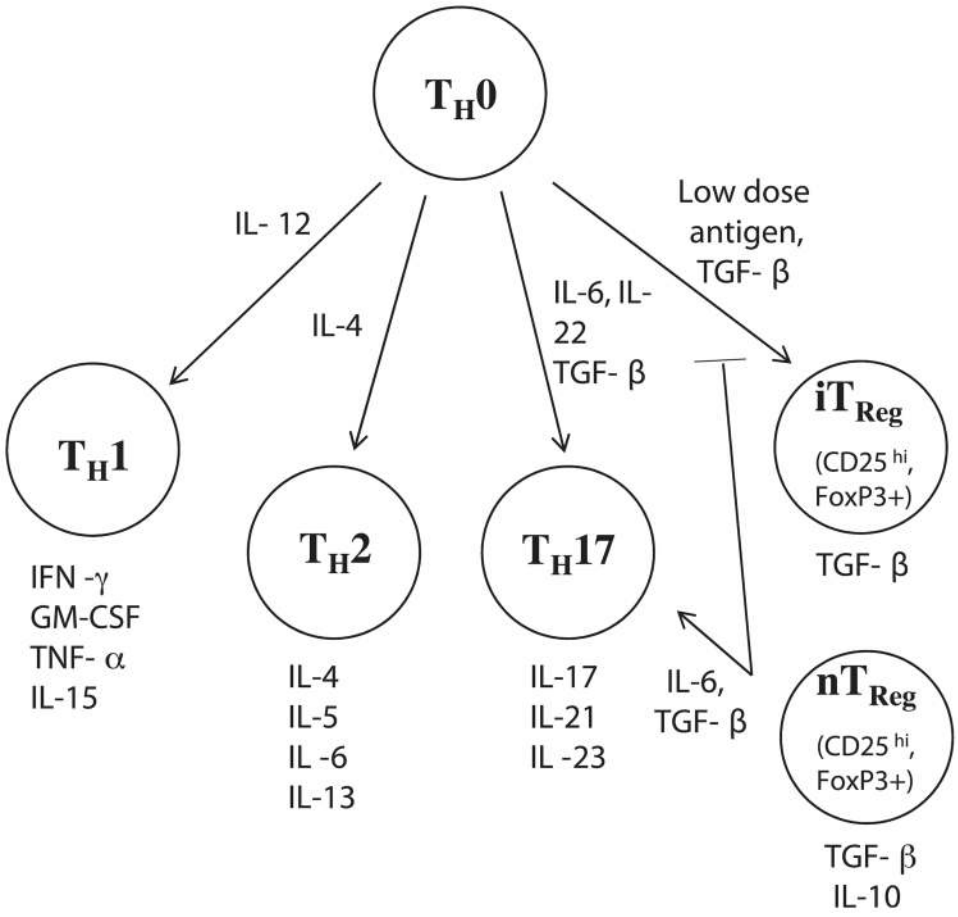

Fig. 4.

Antigen-specific Th cells differentiate under the influence of cytokines into subsets with distinct cytokine profiles and functions. Two classes of Tregs (iTregs and nTregs) produce immunosuppressive effector cytokines that work by juxtacrine and paracrine action. nTregs from the thymus can be influenced by IL-6 and TGF-β to develop into auto-inflammatory Th17 cells, while blocking iTreg development. Other Treg subsets have been described, but are less well established.

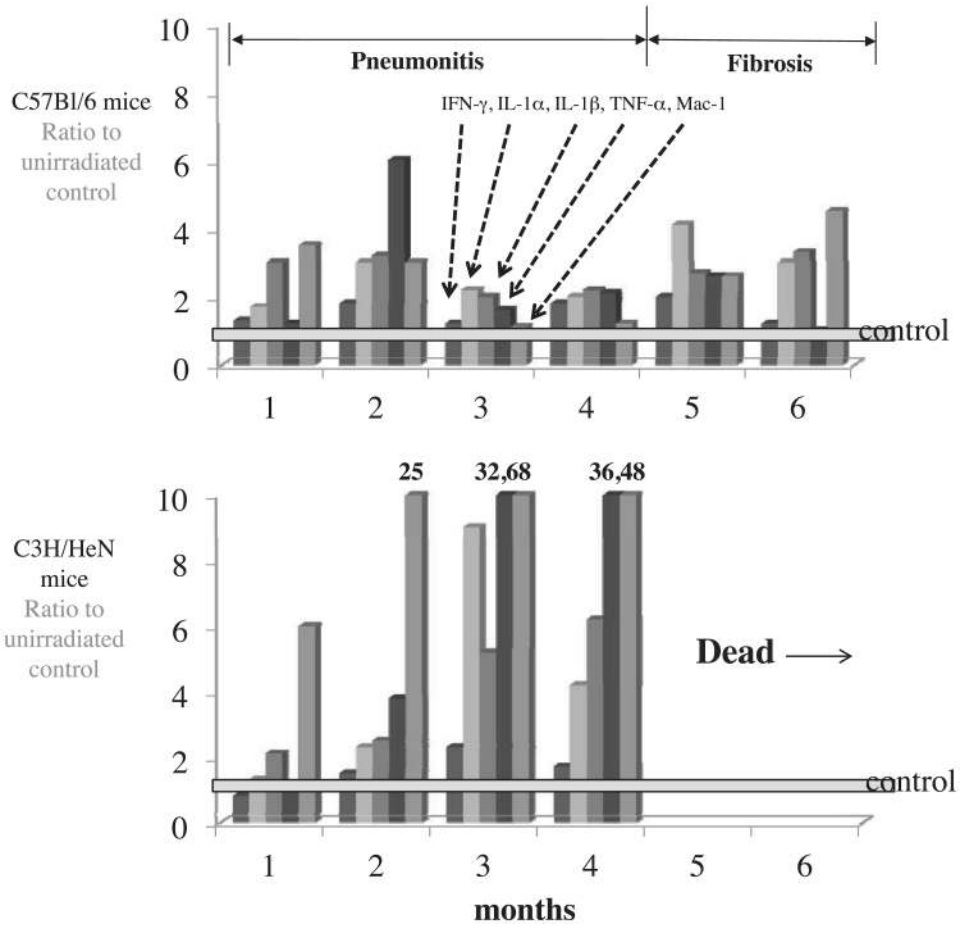

Fig. 5.

Within the first month, the cytokine response of C57Bl/6 mice that develop fibrosis in response to 20 Gy local thoracic irradiation is not markedly different from that of C3H/HeN mice that develop pneumonitis, as assessed by an RNase protection assay of whole lung. However, macrophage (Mac1+ve) infiltration increases with time in irradiated C3H/HeN lungs, followed by large increases in pro-inflammatory cytokines that leads to their death by pneumonitis. C57Bl/6 mice control this pro-inflammatory response, but later develop high levels of IL-6 and TGF-β that lead to lung fibrosis.

Table I

Features of Cytokines

Potent — effective at very low (pM to nM) concentrations.
Transient — mainly produced in response to stimuli. Low “background” levels of some cytokines are present that may be required for homeostasis in some tissues.
Local action — most cytokines influence only cells in the immediate vicinity of production. Some critical variables are the cytokine concentration, formation of cytokine gradients, and presence or absence of extracellular matrix that may modify its persistence and activity. Circulatory levels are normally low, the main exceptions being the hematopoietic factors, e.g., CSF-1 (macrophage colony stimulating factor), EPO (erythropoietin), SCF (stem cell factor/c-Kit), or acute phase reactants e.g., IL-6 or TGF-β that are latent until locally activated. It follows that high serum levels of certain cytokines may reflect pathological processes, but the correlation with tissue events may be poor and they may not be causally involved.
Cell-bound and secreted forms. The potency and functionality of secreted and cell-bound cytokines may be different and the switch may be regulatory. Cell-bound cytokines could interact with adjacent cells through high avidity juxtacrine interactions, while secreted cytokines may function more through autocrine or paracrine mechanisms. Reverse signaling through a ligand may occur, or signaling by soluble complexes through shared receptor subunits, e.g., IL-6 (see text).
Cascadic — responses are propagated by progressive changes in expression patterns of cytokines and their receptors over time. These coordinate to form a cascade that drives responses forward. For example, when specific T cells recognize antigen presented by dendritic cells, the latter can produce IL-1 and TNF-α that aid in formation of the immunological synapse that activates T cells (4) to acquire IL-2R, and so that IL-2 can drive their proliferation, differentiation and activation to produce effector cells that secrete interferon-γ, IL-4, or other effector cytokines.
Pleiotropism — different functions being stimulated depending upon the cell type. For example, TNF-α can be cytotoxic to certain cancer and normal cell types, but causes fibroblasts to proliferate.
Multiple regulatory yin-yang mechanisms. These include:
Mutually agonistic and antagonistic cytokines or receptors or decoys, for example pro- and anti-inflammatory molecules or nonsignaling ligands such as IL-1Ra.
Modulation of function by shedding or internalization of receptors and ligands that alters ligand-receptor interplay, such as soluble TNFRs.
Availability of competing intracellular adaptor proteins, second messengers or other modifiers of signaling. In other words, one receptor can send what appear to be contradictory signals because they are interpreted differently downstream.
Positive and negative feedback loops to terminate or enhance cytokine production. Multiple mechanisms are available. For example, suppressors of cytokine signaling (SOCS) families inhibit STAT activation. Their loss can lead to death due to excessive cytokine production (5). SOCS1 and SOCS3 can differentially modulate intrinsic cellular radiosensitivity (6, 7).

Table 2

Cytokines and Cytokine Receptors

Cytokines
Four α-helix bundle cytokine superfamily - short and long chain families
Short:
IL-2 family: IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, IL-21
IL-3 family: IL-3, IL-5, GM-CSF, M-CSF, SCF
IL-12 family: IL-12, IL-23, IL-27, IL-35
Long:
IL-6 family: IL-6, IL-11, LIF, OSM, CNTF, CT-1, G-CSF, GH, EPO, TPO, leptin
IFN family: Type I (IFN-α, IFN-β), type II (IFN-γ), type III (IL-28, IL-29)
IL-10 family: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26
Beta-Trefoil: IL-1 family - IL-1α, IL-1β, IL-18, FGF
Beta-Sandwich: TNF superfamily
IL-17 conserved cysteine family
TGF-β superfamily
Chemokine superfamily
CC, CXC, XC, and CX_3C families
Cytokine receptors
Type I
Heterodimeric
Using γc to signal: IL-2R, IL-4R, IL-7R, IL-9R, IL-13R, IL-15R
Using βc to signal: IL-3R, IL5R, GM-CSFR
Using gp130 to signal: IL-6R, IL-11R, IL-27R, IL-31, OSMR,CNTFR, LIFR
Homodimeric: G-CSFR, leptinR, EPOR, TPOR
Type II
IFN-α, β, γRs, IL-10R, IL-28R, IL-29R
Ig Superfamily: IL-1R, IL-18R, CSF1R, c-Kit
IL-17R family: IL-17RA to E
TNFR:
Containing death domains: TNF-R1, Fas (CD95), TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R4 (DcR2) and TRAMP (DR3)
No death domains: TNFRII, CD27, CD30, CD40, 4-1BB, RANK
Decoy receptors: TRAIL-R3 (DcR1), DcR3
TGF-βR: TGF-βR1, TGF-βR2
Chemokine receptors
CCR, CXCR, XCR and CX_3CR families

Table 3
Examples of DAMPS and Likely Receptors (in brackets)

DAMPs secreted into extracellular spaces:
ATP, AMP, (P2Rs, NALP3), Adenosine (P1Rs - A1, A2A and B, A3R), advanced glycation end products (RAGE), oxidation products (TLR4, CD36), high mobility group protein B1 (TLR2/4/9, CD44, RAGE), S100 (TLR4, RAGE), monosodium urate (TLR2/4, CD14, NALP3), heat shock proteins (TLR2/4, CD14, CD40, CD91), calcium-binding proteins, beta amyloid (RAGE, NALP3), defensins (TLR4, CCR6), lactoferrin (TLR4), uromodulin (TLR4), surfactant D, ubiquitin (CXCR4).
DAMPS released on cell death:
HMGB1 (TLR2/4/9, CD44, RAGE), dsDNA and chromatin (TLR9), RNA (TLR3), mitochondrial DNA and matrix proteins.
DAMPS from enzymic action on extracellular matrix:
Hyaluronan (TLR2/4, NLRP3, CD44), collagen peptides (CXCR2), elastin/laminin peptides (integrins), fibrinogen/fibronectin (TLR4, integrins), heparan sulfate (TLR4).