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Consensus on the Key Characteristics of Immunotoxic Agents as a Basis for Hazard Identification

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Background: Key characteristics (KCs), properties of agents or exposures that confer potential hazard, have been developed for carcinogens and other toxicant classes. KCs have been used in the systematic assessment of hazards and to identify assay and data gaps that limit screening and risk assessment. Many of the mechanisms through which pharmaceuticals and occupational or environmental agents modulate immune function are well recognized. Thus KCs could be identified for immunoactive substances and applied to improve hazard assessment of immunodulatory agents.

Objectives: The goal was to generate a consensus-based synthesis of scientific evidence describing the KCs of agents known to cause immunotoxicity and potential applications, such as assays to measure the KCs.

Methods: A committee of 18 experts with diverse specialties identified 10 KCs of immunotoxic agents, namely, 1) covalently binds to proteins to form novel antigens, 2) affects antigen processing and presentation, 3) alters immune cell signaling, 4) alters immune cell proliferation, 5) modifies cellular differentiation, 6) alters immune cell–cell communication, 7) alters effector function of specific cell types, 8) alters immune cell trafficking, 9) alters cell death processes, and 10) breaks down immune tolerance. The group considered how these KCs could influence immune processes and contribute to hypersensitivity, inappropriate enhancement, immunosuppression, or autoimmunity.

Discussion: KCs can be used to improve efforts to identify agents that cause immunotoxicity via one or more mechanisms, to develop better testing and biomarker approaches to evaluate immunotoxicity, and to enable a more comprehensive and mechanistic understanding of adverse effects of exposures on the immune system. https://doi.org/10.1289/EHP10800

Introduction

The concept of agents or exposures having properties that confer potential hazards called key characteristics (KCs), was developed during review of the diverse agents identified as established (Group 1) human carcinogens by the International Agency for Research on Cancer (IARC).[1] It was recognized that, although these agents are diverse and act through multiple mechanisms, they share common properties, or KCs, that could be used as an organizing principle for research and synthesis and to support the evaluation of agents of unknown carcinogenic potential.[1] This concept has provided a framework to use to systematically evaluate known and suspected carcinogens based on mechanisms by which known human carcinogens act, has allowed gaps in knowledge to be identified, and has guided the design of cellular and molecular assays that can better predict carcinogenicity in humans.[1,2] The KCs of human carcinogens are now widely used by various environmental[3–5] and pharmaceutical[6] regulatory agencies and form the basis for the evaluation and integration of mechanistic data in the risk assessment process. In response to a U.S. National Academies of Sciences, Engineering, and Medicine report that recommended extending the approach beyond cancer hazard identification,[7] the KCs of endocrine-disrupting chemicals,[8] reproductive toxicants,[9,10] hepatotoxicants,[11] and cardiovascular toxicants[12] have recently been published. Herein, we describe the application of the KC concept to immunotoxic agents. These KCs, along with complementary information, provide a framework to identify and characterize compounds that may have undesired effects on immune function. In addition, it is important to establish KCs for different classes of toxicants that induce major adverse health effects so that shared mechanisms can be considered collectively.

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Many aspects of the mechanisms through which xenobiotics affect the immune system are well understood, and the cellular and molecular targets for these agents have been described. Numerous pharmaceuticals are designed to modulate the immune system for therapeutic benefit, whereas pharmaceuticals designed for other purposes,[13] as well as certain chemicals found in the environment and workplace, may adversely affect immune function.[14–17] Immune suppression, a reduction in the capacity of the immune system to respond effectively to foreign or tumor-associated antigens, can lead to serious clinical consequences, including reduced resistance to infection and neoplasia. Epidemiological data from patients with congenital immunodeficiencies,[18,19] virally induced immunodeficiencies [e.g., human immunodeficiency virus (HIV)-mediated],[20–22] and from patients treated with immunosuppressive therapies (e.g., transplant rejection prevention therapies)[23,24] clearly demonstrate that significant immunosuppression increases the risk of both infection and cancer. Multiple pathways are involved in evading innate and adaptive immune responses, with a broad spectrum of agents displaying the potential to adversely influence immunosurveillance.[25]

In addition to dampening immune responses, an agent or drug can also induce immune dysregulation, leading to inappropriate immune stimulation. Consequences of overly active immune responses include chronic inflammation, allergic sensitization, or autoimmunity.[17] Chronic inflammation, induction of which has been described as a KC of carcinogens because it can promote tumor development,[1] and inappropriate immune stimulation may also lead to cytokine release syndrome (CRS),[26] a systemic inflammatory response.[17] In allergic hypersensitivity, the immune system responds to chemically modified (nonself) compounds (haptens) as part of a specific immune response. The most common health consequences include respiratory tract allergies (e.g., asthma, rhinitis) or allergic contact dermatitis (ACD).[17] Autoimmune disease occurs when the immune system recognizes host tissue as foreign and mounts an immunologic response against it, resulting in structural or functional damage.[27] Clinical manifestations of inappropriate immune stimulation are very diverse, with systemic reactions such as anaphylactic shock or local reactions such as erythema and edema, and involve a diversity of target organs.[28]

In this commentary, we have used this mechanistic knowledge to develop a consensus-based synthesis of scientific evidence to identify the KCs of immunotoxic agents, defined as substances that can alter one or more immune functions, resulting in an adverse effect for the host. Thus, an immunotoxic agent may exhibit one or more of the KCs. We provide examples demonstrating the use of these KCs to characterize the toxicity of various agents, recognizing that some substances may initiate cascades of events and demonstrate multiple characteristics that define immunotoxicants. We provide suggestions for methods to assess how unknown agents may exhibit specific KCs. An understanding of how substances cause immunotoxicity earlier in the drug discovery or hazard assessment process will allow us to develop better tests to evaluate immunotoxicity in humans. Together the KCs and associated tests will enable a more comprehensive mechanistic understanding of the adverse effects of exposures on immunity.

Methods

We assembled a group of 18 experts with broad-ranging knowledge of the immune system and immunotoxicity, hazard evaluations and risk assessments, pharmaceutical safety evaluation, and clinical immunology, with the goal of developing KCs of immunotoxic agents. The group met biweekly by video conference from September 2020 to April 2021. Lists of possible KCs of immunotoxicants, based on known examples and expert knowledge, were prepared. In developing the KCs, the authors considered both traditional immunotoxicology literature for chemicals, metals, oxidant gases, etc. and the published literature on therapeutics (e.g., compounds designed to be immunosuppressive and therapeutics that have unintended immunomodulatory activity). Criteria for potential KCs included published information on chemical characteristics or mechanisms by which substances may act as immunotoxicants that were distinct and nonoverlapping and broad enough to be demonstrated by multiple chemicals/examples, and for which plausible empirical evidence exists to support that a substance, or class of substances, affects immune cell functions. The preliminary lists were consolidated, and a set of 10 KCs of major relevance was identified through group discussion and consensus. For each KC, a description was drafted by a subgroup comprising a primary author and one or two secondary authors/reviewers. The descriptions were subsequently reviewed and finalized by all authors and group consensus and a composite illustration was prepared (Figure 1).

We also considered the fact that two of the KCs of carcinogens involve broad immune-mediated effects, namely, "induces chronic inflammation" and "is immunosuppressive."[1] We elected not to include chronic inflammation (and other broad descriptors of overly active responses) or immunosuppression as KCs of immunotoxicants because they are consequences of immunomodulatory agents and because there are multiple KCs that can lead to enhanced or poorly controlled immune responses or immunosuppression.

We developed two examples to illustrate the application and context of the KCs of immunotoxicants, outlining which KCs were exhibited by the immunosuppressive drug cyclosporin A (CsA; Figure 2) and aryl hydrocarbon receptor (AhR) ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; Figure 3). To explore the use of these KCs of immunotoxicants, we also examined them in the context of developmental immunotoxicity, a well-recognized sensitive window for immune effects, and considered applications of the KCs to hazard identification, risk assessment, and clinical practice (Figure 4).

Descriptions of the Key Characteristics of Immunotoxic Agents

The 10 KCs of immunotoxicants identified by consensus and reflective of current scientific evidence were as follows: 1) covalently binds to proteins to form novel antigens, 2) affects antigen processing and presentation, 3) alters immune cell signaling, 4) alters immune cell proliferation, 5) modifies cellular differentiation, 6) alters immune cell–cell communication, 7) alters effector function of specific cell types, 8) alters immune cell trafficking, 9) alters cell death processes, and 10) breaks down immune tolerance. All 10 are described in detail below. We acknowledge that the 10 KCs will likely evolve with scientific knowledge and that additional KCs could be added in the future. An illustration of how various classes of exposures may exhibit one or more KCs leading to hypersensitivity, inappropriate enhancement, immunosuppression, or autoimmunity is provided (Figure 1).

KC1: Covalently Binds to Proteins to Form Novel Antigens

Haptenization defines the reaction of a compound (hapten) with a carrier protein to form a conjugate able to stimulate an immune response. This reaction is considered the molecular initiating event that triggers chemical sensitization.[29] It is believed to be central to chemical-induced sensitization in both skin and respiratory allergy[30] and for low-molecular-weight allergenic drugs.[31] Some small molecules, including certain heavy metals and medicines, can be associated with autoimmune-like reactions.[32]

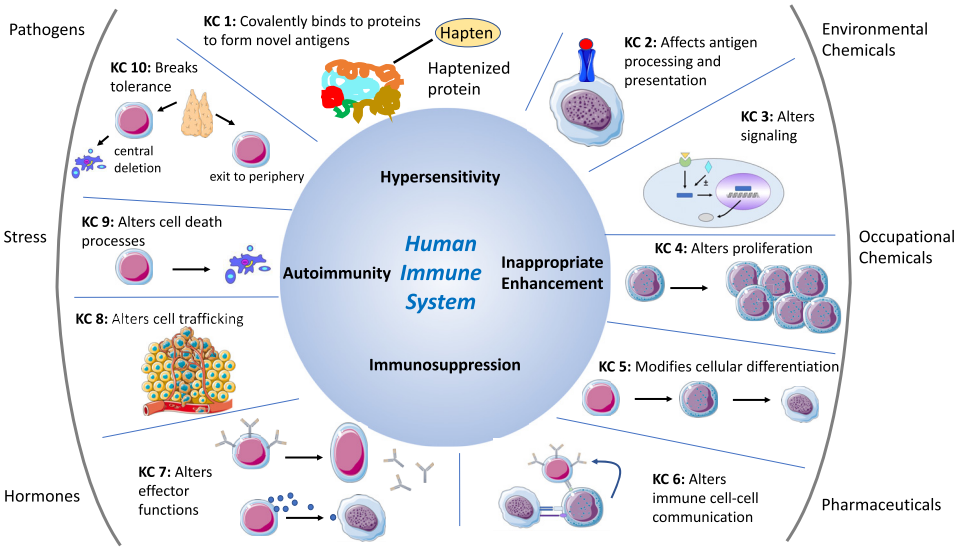

Figure 1. The key characteristics (KCs) of immunotoxicants. Various classes of exposures (outside) may exhibit any one or more of the 10 identified KCs (middle) leading to hypersensitivity, inappropriate enhancement, immunosuppression, or autoimmunity (inside). The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

Chemical allergy refers to hypersensitive immune responses to small molecules. Chemical haptens can be divided into three classes: *a*) direct haptens; *b*) pro-haptens, which require metabolic activation; and *c*) prehaptens, which spontaneously oxidize to form haptens.[29,30] Typically, chemical allergens are small molecules (<500 Da), which, through the covalent binding of the parent compounds or their metabolites to carrier proteins, become immunogenic.[33] The complex formation is related to electrophilic reactivity and hydrophobicity of the allergen. In this form, they are recognized by antigen-presenting dendritic cells (DCs). In response to allergens, DCs differentiate into mature immunostimulatory cells by up-regulation of costimulatory molecules such as CD80, CD86, and CD40 and adhesion molecules such as CD2, CD54, and CD58 and produce the cytokines, including interleukin (IL)-1β, IL-8, IL-12, and IL-18, necessary for T-cell activation.[29,33] The specific immune response takes place in draining lymph nodes, where DCs migrate and stimulate the activation of hapten-specific T cells and the generation of effector cells. Following stimulation, clonal expansion of T cells able to react to the antigen occurs resulting in allergic reactions.[29,30]

The acquisition of sensitization in chemical allergies occurs in two phases: *a*) the induction phase, when the hapten combines with a protein to form a conjugate that leads to the clonal expansion of allergen-specific B- and T-cell populations, and *b*) the elicitation phase upon reexposure to the same antigen, when an inflammatory response is elicited that can lead to the clinical manifestation (e.g., allergic asthma, ACD), or systemic hypersensitivity.[29,30,33] ACD is a delayed-type hypersensitivity reaction caused mainly by the generation of $CD8^+$ Tc1/Tc17 and $CD4^+$ Th1/Th17 effector T cells as a result of repeated exposure to an allergen, primarily on the skin.[34] Common contact allergens include metals (nickel, cobalt, chromium), preservatives (methylisothiazolinone), and fragrances (cinnamal, limonene), whereas among chemical-induced type I hypersensitivities, anhydrides (phthalic anhydride), platinum salts, and isothiocyanates are recognized.[35] Beta-lactam antibiotics are the most common pharmaceutical agents implicated in drug allergy. They can induce multiple types of hypersensitivity reactions, depending on the route of administration; allergy to beta-lactam antibiotics is more commonly seen after parenteral than oral administration.[36,37]

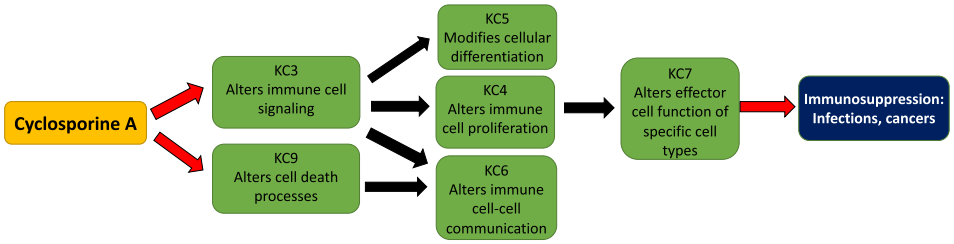

Figure 2. Cyclosporine A (CsA) exhibits six key characteristics (KCs) of immunotoxicants. CsA is a widely used immunosuppressive drug whose mode of action has been well characterized in humans and experimental animals.[52] Its main therapeutic indication is the treatment and prevention of organ rejection in kidney, liver, and heart allogeneic transplants.[52] As a consequence of its immunosuppressive activity, infections and cancer are observed in long-term treated patients.[23,24,53] CsA acts on key mechanisms needed for many aspects of the immune response and exhibits six KCs of immunotoxicants as detailed in the respective KC descriptions. KC3: In T lymphocytes CsA binds to cyclophilin A, forming a complex inhibiting the phosphatase activity of calcineurin A and, consequently, the translocation of the nuclear factor of activated T cells (NFAT) transcription factor into the nucleus. The absence of NFAT translocation alters the transcription of key genes implicated in T cell proliferation and function (IL-2, IL-4, CD40 ligand). CsA also affects the activities of the AP-1 and NF-κB transcription factors. KC4: Via its effect on NFAT, CsA inhibits IL-2 synthesis and, consequently, T-cell proliferation. KC5: The effects of CsA on transcription factors and key molecular mechanisms lead to altered cytokine production, T-cell polarization, B-cell differentiation in plasmocytes, and cytotoxic T lymphocyte activation. KC6: Inhibition of cytokine production alters CsA-mediated $CD4^+$ T lymphocytes help to B lymphocytes. KC7: Alteration of cell differentiation and cell–cell communication lead to altered antibody production by plasmocytes and cell killing by cytotoxic T lymphocytes. KC9: The mitochondrial permeability transition pore (mPTP) involved in stress and calcium cell death is sensitive to CsA. Note: AP-1, activator protein 1; IL, interleukin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.

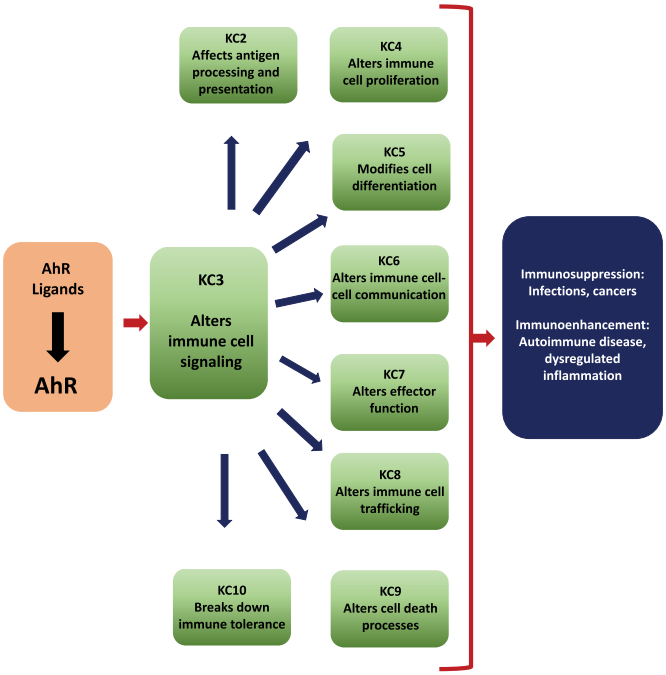

Figure 3. AhR ligands exhibit nine key characteristics (KCs) of immunotoxicity. The aryl hydrocarbon receptor (AhR) is a transcription factor that is broadly expressed, including in immune cells. AhR ligands, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and FICZ, are considered immunomodulators because they have the potential to produce immune suppression or immune enhancement through several of the KCs.[77] KC2: Attenuation of dendritic cell (DC) ability to activate naïve T cells and changes to the expression of cell surface receptors may contribute to KC8.[112,172–176] KC3: Effects are mediated via AhR, leading to changes in gene expression and cell signaling.[112,173,177–179] KC4: AhR ligands reduce T-cell clonal expansion[56,57,180] and impair proliferation of B cells[181–184] and hematopoietic stem and progenitor cells (HSPCs).[185–187] Effects on proliferation can contribute to KC5, KC6, and KC7. KC5: AhR ligands skew T-cell differentiation, reduce B cell differentiation, and affect context-dependent alteration of monocyte differentiation.[56,58,180,181,184,188–190] Effects on differentiation can contribute to KC6 and KC7. KC6: AhR ligands induce modulation of cytokines, chemokines, and adhesion molecules.[191–194] Perturbation of cell–cell communication can contribute to all other KCs. KC7: AhR ligands were shown to inhibit B-cell activation and antibody production,[102,182,183] T-cell activation, and induce cytotoxicity of $CD8^+$ T cells.[89,169,180,195] Alterations in effector cell functions can contribute to KC4 and KC5. KC8: Neutrophil accumulation in inflamed tissues, and reduced DC trafficking,[176,196–199] can contribute to KC2–5. KC9: Thymocyte apoptosis[166] and B cell death[200] may contribute to KC4, KC7, and KC10. KC10: Enhanced Treg cell frequency and tolerogenic DCs[56,58,201–203] can contribute to KC2 and KC7. Note: DC, dendritic cell; FICZ, 6-formylindolo[3,2-b]carbazole; Treg, regulatory T cell.

Currently, there are >4,000 substances that are identified as chemical allergens, including fragrances, hair dyes, preservatives, nickel and other metals, and drugs.[35,38] Even if the underlying mechanisms are not fully understood, some low-molecular-weight sensitizers can induce immunological responses that trigger asthma or other symptoms in the respiratory tract following repeated exposure, often associated with specific IgE production.[33] Respiratory sensitization is a serious health issue in occupational medicine, with potentially life-threatening consequences owing to possible anaphylactic shock.[39]

KC2: Affects Antigen Processing and Presentation

Antigen presentation by DCs, macrophages, monocytes, and B cells is essential for T-cell immune responses and adaptive immunity. Three steps are involved: *a*) antigen penetration and internalization into the antigen-presenting cell (APC) using clathrin-mediated endocytosis, phagocytosis, or micropinocytosis; *b*) antigen processing, where proteins are mainly degraded into small peptides by cytosolic proteases; and *c*) antigen presentation, where peptides are transported and displayed on cell surfaces bound to major histocompatibility complex (MHC) molecules.[40] $CD8^+$ T cells recognize protein-derived peptides (antigen) in association with MHC class I molecules, whereas $CD4^+$ T cells recognize peptides (antigen) bound to MHC class II molecules.[40] Altering any of these three steps can lead to either immunosuppression or autoimmune and hypersensitivity reactions.[17] Moreover, some agents can directly bind to Toll-like-receptors (TLR), contributing to DC maturation and MHC class II expression. Indeed, nickel is a well-known TLR4 agonist, explaining why it is such a potent allergen.[41]

Many agents are known to affect multiple steps of this process. Chlorpromazine, a well-known antipsychotic (neuroleptic) agent, blocks clathrin-dependent processes and inhibits micropinocytosis and antigen penetration into APCs.[42,43] Chloroquine (anti-malaria drug) inhibits lysosomal acidification and MHC class II antigen presentation.[44] In addition, selective inhibitors of cathepsin S (cancer immunotherapeutics) interfere with the processing of the MHC class II invariant chain li and reduce presentation of auto-antigens.[45] Inhibitors of aspartyl proteases can alter the presentation of encephalitogenic myelin basic protein epitopes, altering immune tolerance.[46] Inhibiting proteases can modulate specificity of epitope generation and induce the generation of other epitopes that trigger (different) self-reactive T cells, inducing autoimmune responses.[46]

Glucocorticoids can inhibit the activation of DCs by reducing the levels of MHC II molecules.[47] T-2 toxin, a mycotoxin associated with alimentary toxic aleukia, reduced antigen presentation and MHC II expression in a mouse model of dermal hypersensitivity, resulting in decreased inflammatory responses at the site of application of the sensitizing agent.[48] Similar reductions in MHC II expression were observed in rodent Langerhans cells exposed to T-2 toxin *in vitro*.[48]

The antiviral abacavir binds to human leukocyte antigen (HLA)-B*57:01 (human MHC) and changes the shape of the antigen-binding cleft, thereby altering the repertoire of endogenous peptides that can bind HLA-B*57:01 and provoking alteration in immunological self.[49] The resulting altered self activates abacavir-specific T cells, thereby driving polyclonal CD8 T-cell activation and a systemic reaction that manifests as abacavir hypersensitivity syndrome.[49]

The pharmacological interaction with immune receptor (p-i) concept relates to a mechanism of drug hypersensitivity that represents an off-target interaction of drugs with immune receptors [HLA and/or T-cell receptor (TCR)].[50] These pharmacological interactions appear to result in altered antigen presentation, occurring through noncovalent interactions that trigger TCR signaling outside of normal stimulatory pathways (i.e., in the absence of costimulation).[50] Clinically severe immune reactions affecting mostly skin and liver have been observed following treatment with drugs such as allopurinol, sulfomethoxazole, or carbamazepine owing to an unorthodox, alloimmune-like stimulation of T cells.[50] It has been suggested that the p-i concept be incorporated into preclinical risk assessment strategies.[50]

KC3: Alters Immune Cell Signaling

Cell signaling describes the molecular process by which cellular receptors are activated and signals transmitted through the cell to elicit responses, including transcription, enzymatic activity, cell proliferation, survival, activation, migration, and differentiation. One of the best-characterized signaling cascades in immune cells is the TCR pathway.[51] Following recognition of antigenic peptide and MHC, the TCR/CD3 complex, in cooperation with

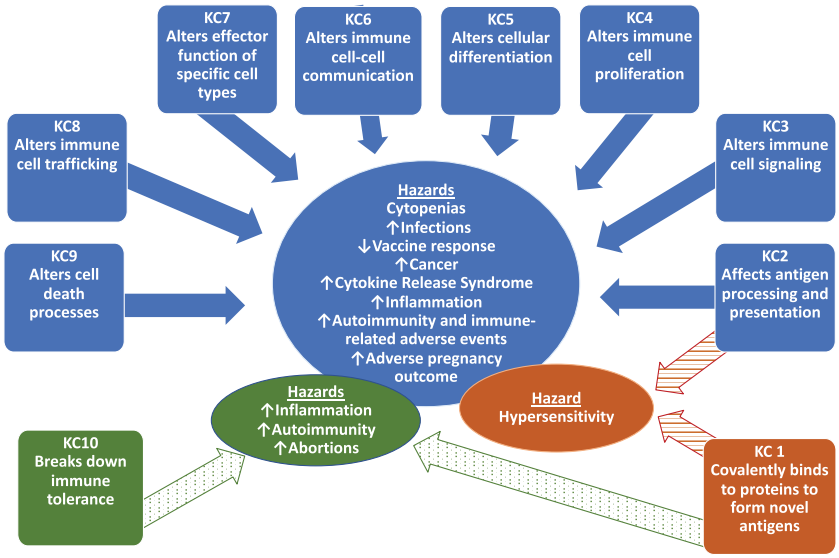

Figure 4. Implications of the Key Characteristics (KCs) of immunotoxicants for understanding disease. Each of the KCs may contribute to a health hazard or clinical disease, with KC1 and KC2 being the predominant mechanism for increased hypersensitivity (orange shading and arrows with a horizontal stripe pattern), and KC10 contributing mainly to increased risk of autoimmunity, inflammation, and recurrent miscarriage (green shading and arrows with a dotted pattern). The remaining KCs, KC2–7, jointly contribute to multiple outcomes including cytopenias and increased infection. (indicated by blue shading and solid arrows). In the authors' opinion, the KCs can be used to protect human health by enhancing understanding of the pathogenesis of related disease processes and by informing the development of less immunotoxic medicines and consumer products. The KCs can also be used as an organizational framework that provides mechanistic insight for identifying and evaluating risks to the human immune system from environmental chemicals.

costimulatory molecules, such as CD28, transduces signals through kinases and second messengers, such as calcium ions (Ca^{2+}) and diacylglycerol, culminating in activation and translocation of transcription factors [e.g., nuclear factor of activated T cells (NFAT), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and activator protein 1 (AP-1)], which drive expression of a number of genes critical for effective immune responses.[51] Disruption of immune cell signaling pathways by molecules that act directly on signaling components can lead to profound immunosuppression, whereas poorly controlled activation can lead to pathophysiological hyperinflammation. The drug CsA is a potent immune cell signaling disruptor and acts by inhibiting calcineurin, a critical intermediary between the Ca^{2+} sensing protein calmodulin and activation of NFAT[52] (Figure 2). CsA prevents the translocation of NFAT, thereby blocking the transcription of cytokine genes, including IL-2. CsA also blocks the Jun N-terminal kinase and p38 kinases in TCR signaling pathways to further inhibit T-cell activation.[52] Although calcineurin inhibitors are important medicines in organ transplantation, graft-vs-host disease, autoimmunity, and inflammation, their clinical use must be managed carefully owing to the increased risk of infections and neoplasia.[53]

The AhR also mediates immunomodulatory signals that can lead to immunotoxicities depending on the ligand, cell type, and microenvironment (Figure 3)[54]; however, it uses a mechanism distinct from CsA. The AhR is a transcription factor found in the cytosol in a transcriptionally inactive state. Upon binding to a ligand, AhR translocates to the nucleus and controls the transcription of genes that contain aryl hydrocarbon–responsive elements.[54,55] A variety of structurally distinct AhR ligands have been identified from environmental, microbial, dietary, and endogenous sources that can modulate immune responses in health and numerous disease states. The best-studied AhR ligand is the pollutant TCDD, which has a high affinity for AhR and potently suppresses adaptive immunity by influencing the function of numerous cells, including T cells, DCs, and B cells.[54] Although other AhR ligands may induce effects similar to TCDD, they also elicit distinct consequences, leaving open questions as to precisely how AhR ligands modulate immune cell functions[56] (Figure 3). Distinct outcomes of AhR activation depend on the ligand, antigenic challenge, and immune cell type, as well as the microenvironmental context and potentially cell-type specific interactions with transcriptional coactivators.[56–58] For example, in some model systems, the tryptophan metabolite 6-formylindolo[3,2-b] carbazole (FICZ) has the opposite effect of TCDD, enhancing T-follicular helper cells and stimulating pro-inflammatory Th17 and IL-22 responses.[56,57] These opposing effects can lead to immunosuppressed states and exacerbated inflammatory diseases, respectively.

Excessive activation of immune cell signal transduction pathways can lead to severe diseases, such as CRS. CRS is a self-perpetuating inflammatory cytokine cascade thought to be initially triggered by cytokines released from T cells.[26] Some examples of agents that induce CRS include TGN1412 (an anti-CD28 superagonist monoclonal antibody), OKT3 (muronomab anti-CD3), adoptive T-cell therapies, and nonprotein-therapeutics such as oxaliplatin.[59] Signaling pathways that activate NF-κB and interferon response factors drive production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), IL-1, IL-6, and chemokines. These cytokines and chemokines can activate innate immune cells and endothelial cells to produce more inflammatory cytokines and cause tissue damage, perpetuating the systemic inflammatory cycle.[26] The uncontrolled amplification of inflammatory cytokines produces conditions ranging from mild flu-like symptoms to high-grade fever, fluctuations in blood pressure, capillary leakage, and severe hypoxia, as well as multi-organ failure.[26]

KC4: Alters Immune Cell Proliferation

Cell proliferation plays an integral role in the immune response, and changes to this process may lead to immune dysfunction. Perhaps the most profound effects can occur through decreases in hematopoietic stem and progenitor cell (HSPC) proliferation,

which can result in the depletion of entire lineages of immune cells (e.g., neutropenia, lymphopenia).[60] Some forms of cancer therapy, including radiation therapy and many chemotherapeutic agents, which are selected because they inhibit the proliferation of cancer cells, also inhibit the proliferation of HSPCs.[60] Myelotoxicity, which can manifest as HSPC cytostasis or cytotoxicity, depending on the agent and level of exposure, results in neutropenia and an increased susceptibility to infection and is the most common dose-limiting toxicity in cancer therapy with classical chemotherapeutic agents or radiotherapy.[60] The solvent benzene, a frequent soil and groundwater contaminant, is an example of a known nontherapeutic myelotoxic agent.[61] Benzene induces genetic and epigenetic abnormalities in hematopoietic stem cells (HSCs), produces genomic instability, dysregulates stromal cells, induces apoptosis of HSCs and stromal cells, and alters their proliferation and differentiation.[62] These effects—modulated by oxidative stress, AhR dysregulation, and reduced immunosurveillance—lead to a dysregulated immune response, hematotoxicity, and leukemia.[62]

Cell proliferation is fundamental, not only for maintaining the basic infrastructure of the immune system, but also for enabling adaptive immune responses. During an immune response, antigen-specific lymphocytes clonally expand to produce enough effector cells to address a pathogenic challenge.[40] If lymphocyte proliferation is inhibited, then immunosuppression is a common outcome. It is also possible for an agent to enhance the level or prolong the duration of immune cell proliferation, which could contribute to immune-mediated pathologies, such as the inadvertent destruction of healthy tissues. Lymphocyte proliferation is so closely linked with immune function that rates of lymphocyte proliferation are commonly measured and considered a reliable index for identifying and comparing the relative potencies of immunotoxic agents.[63]

Immunotoxicants can inhibit cell proliferation through a wide variety of mechanisms, including direct impacts on the mitotic spindle (e.g., vinca alkaloids[64]) or alterations to gene expression (e.g., AhR ligands), cell cycle regulatory machinery (e.g., arsenic[65]), as well as effects on cell signaling, cytokine production (e.g., CsA[52]), and responses to cytokine stimulation (e.g., sirolimus[66]). Although effects on immune cell proliferation are generally considered to be most important in the context of immunosuppression, there is also evidence to suggest that lymphocyte proliferative capacity can be modulated in either direction as a result of developmental exposure to some immunotoxic agents (e.g., endocrine-disrupting chemicals), although the mechanisms by which this occurs are poorly understood and could be closely linked with effects on cellular differentiation.[67,68] It is also unclear whether enhanced immune cell proliferation could impact processes associated with immunostimulation, such as hypersensitivity or autoimmunity.

KC5: Modifies Cellular Differentiation

The ability of immune cells to rapidly differentiate in response to micro- and macro-environmental cues, and to maintain this capacity across the lifespan, is vital to the function of the immune system. Regulated differentiation is essential for producing new immune cells from HSPCs, thus a key aspect of the formation of new immune cells at steady-state and in response to infection or injury involves regulated differentiation.[40] In addition, once developmentally matured, many types of immune cells exist in a poised state, requiring additional differentiation before they are able to carry out cell type–specific functions. Adding another layer of complexity, some immune cells, notably T cells—but also monocytes, macrophages, and DCs—are able to nonpermanently differentiate into different subclasses of effector cells to carry out distinct functions.[69–71] This allows plasticity in the repertoire of immune defenses. Yet this also means that agents that impinge on a range of essential cellular processes can influence immune cell differentiation. Moreover, disrupted immune cell differentiation can lead to suppression or inappropriate enhancement of immune function.

The ability to modify immune cell differentiation is the foundation of many therapeutic agents, such as CsA and tacrolimus, that interrupt signaling pathways and prevent the expression of genes encoding factors that regulate cellular differentiation.[72,73] In some instances, small molecules affect immune cell differentiation in a manner that is unintended and undesirable. Benzene, a human leukemogen and immunotoxic agent, may dysregulate innate immunity by disrupting myeloid cell differentiation and suppress adaptive immunity via impeding lymphoid differentiation and reducing mature peripheral T and B cell numbers.[74,75] Interestingly, the immunotoxic consequences of benzene exposure depend on dose and duration. Higher-dose/shorter-term exposures are hematotoxic and impair cell proliferation, which manifests as pancytopenia and aplastic anemia, whereas lower-dose/longer-term exposures to benzene skew hematopoietic cell differentiation and contribute to leukemia.[61,62,76] There are other examples of chemicals that modify differentiation in dissimilar ways, such as TCDD and FICZ, which are small molecules that bind to the AhR. Depending upon the model system and cell type, AhR ligands can cause immune cells to differentiate along a different trajectory (Figure 3).[56,57,77] For example, AhR agonists modulate $CD4^+$ T-cell differentiation; yet the direction of change is not the same for all $CD4^+$ T-cell subtypes or for all AhR ligands.[56,58,78,79] Although molecular targets and mechanisms likely vary, emerging evidence from AhR ligands,[80] as well as pollutants such as trichloroethylene[81] and mercury,[82] indicates that some exposures can modify epigenetic regulatory mechanisms in immune cells, which can also skew differentiation.

KC6: Alters Immune Cell–Cell Communication

Immune cell homeostasis is tightly regulated via cytokines and other soluble factors (e.g., interferons, interleukins, chemokines, TNFs) produced by a variety of cell types.[83] These include not only immune cells, such as DCs, monocytes/macrophages, T and B cells, and mast cells, but also nonimmune epithelial, endothelial, and stromal cells. Homeostasis is also regulated by receptor/ligand interactions and is therefore dependent on the expression of multiple receptors. These soluble or surface (some intracellular) molecules are critical in the regulation of activation, proliferation, survival, and effector function of all immune cells and regulate interactions of immune cells with nonimmune cells.[84]

An excessive inhibition of cytokine production or activity may cause immunosuppression (decreased antibody production, decreased cytolytic activity) that can be associated with an increased risk of infection[85] or cancer,[13] whereas an excessive production of cytokines can cause adverse inflammation, vascular leakage syndrome, or CRS.[86] Many pharmacological agents (e.g., glucocorticoids[87] and CsA[88]) and chemicals (e.g., TCDD[89]) have immunosuppressive properties directly related to the inhibition of cytokine production or activity. The role of cytokines and the consequences of cytokine inhibition are also illustrated by the anti-cytokine or cytokine receptor antibodies developed as anti-inflammatory agents. Conversely, immunomodulating agents can increase production of cytokines via interaction with pattern-recognition receptors such as TLRs (e.g., imiquimod, resiquimod, CpG oligonucleotides, lipopolysaccharide, ssRNA and dsRNA viruses) or inflammasomes (e.g., croton oil or sensitizers such as nickel or dinitrofluorobenzene[90]). Activation of surface receptors such as CD28 (TGN1412)[91] or CD3 (OKT3,

blinatumomab)[92] by targeted therapeutics may lead to excessive cytokine production by T-cells and CRS.

Immune cells also communicate via noncytokine-mediated inhibitory or activating receptor–ligand interactions, which play a critical role in immune homeostasis. In particular, T-cell activation is tightly regulated by cell–cell interactions with APCs.[71] Proper T-cell activation requires TCR/MHC interactions and the appropriate balance between costimulatory (e.g., CD28/B7; ICOS/B7RP1; 4-1BB/4-1BBL; OX-40/OX-40L) and inhibitory (e.g., CTLA-4/B7; PD1/PDL1-PDL2; TIGIT/nectins) signals. The purposeful disruption of signals provided by these interactions has been extensively leveraged therapeutically and illustrates how interfering with these pathways can lead to either immunosuppression (e.g., abatacept),[93] or immune-related adverse events[85] linked to increased T-cell activation (e.g., pembrolizumab, ipilimumab, nivolumab).[94,95] It can be hypothesized that xenobiotics impacting these pathways would be associated with immunotoxicity.

KC7: Alters Effector Function of Specific Cell Types

Proper immune function is the result of orchestrated responses mediated by mechanisms involving cellular and soluble effectors. Effector function is exhibited by innate immunity cells and functions [myeloid cell–mediated phagocytosis, cytokine production, and respiratory burst; natural killer (NK) cells and target cell killing] and acquired immunity cells and functions [plasma cells, effector B cells and antibody production, helper T cells and cytokine production, cytotoxic T lymphocytes (CTLs) and target cell killing].[40]

Phagocytosis plays an important role in antibacterial immunity. Agents that impact phagocytosis include tetracyclines, bacitracin, antimalarial drugs,[96] thimerosal, and *p*-nitrophenyl methyl disulfide,[97] whereas agents that interfere with intracellular killing of pathogens by phagocytes include wortmannins,[98] trimethoprim, and sulfamethoxazole.[99]

NK cell effector function plays an important role in antiviral immunity and surveillance of tumors and is controlled by the balance of activation and inhibitory signals received through NK receptors in response to interactions with ligands such as MHC class I molecules, MHC class I-like molecules, and non-MHC–induced stress-related surface proteins.[100] Agents that impact NK activity include pharmaceuticals such as chemotherapeutic agents, CsA, dexamethasone, ustekinumab, tofacitinib,[100] and antibodies directed to NKG2A or other membrane proteins expressed on NK cells, as well as chemicals such as TCDD.[101]

Antibody or immunoglobulin production and secretion by B cells is a key component of humoral immunity against pathogens. The effector mechanisms mediated by antibodies involve antigen-specific recognition via a fragment antigen-binding domain and interaction with effector cells (phagocytes, NK cells) via the crystallizable fragment domain.[40] Molecules impacting B-cell function include those causing depletion of B cells, such as rituximab or blinatumomab, and agents interfering with B cell activation, immunoglobulin isotype switching, and/or antibody production, such as TCDD,[102–104] iron,[105] and mercury.[106] Other heavy metals, such as cadmium, have been shown to alter both the amount and specific immunoglobulin isotype produced by human peripheral blood mononuclear cells *in vitro*.[107,108]

The main effector function of helper T cells consists of producing cytokines that contribute to B cell activation, antibody production, and class-switching, as well to support cytotoxic T-cell function. Agents that interfere with helper T-cell function include those that block T-cell activation and proliferation (muromonab-CD3, CsA, tacrolimus, abatacept, sirolimus).[88] Environmental agents have also been associated with alterations in helper T-cell functions, including AhR ligands[56,78] (also see Figure 3), heavy metals,[109] and volatile organics, such as trichloroethylene.[81] CTLs exhibit an antigen-specific effector function mediated via recognition of peptide–MHC Class I complexes and lead to the death of target cells through the release of lytic granules (perforin and granzymes) or receptor–ligand binding (Fas/FasL).[110] CTL activity is impacted by agents such as dexamethasone, tacrolimus, and CsA,[111] as well as by environmental chemicals such as dioxins and polychlorinated biphenyls.[112] Although inhibition of individual effector functions may lead to immune suppression and the increased risk of disease, impacting multiple functions at one time may increase the severity of the outcome. For example, in a rodent model of latent viral reactivation, blocking CTL or NK cell function alone resulted in minimal latent cytomegalovirus reactivation. However, when both CTL and NK effector function were blocked simultaneously, viral reactivation was increased to 80%.[113]

KC8: Alters Immune Cell Trafficking

A unique aspect of an effective immune response is the ability of immune cells to travel (i.e., traffic) to the site of insult. For example, circulating innate cells respond to chemotactic gradients established by immune and nonimmune cells producing chemokines locally in response to a pathogen, danger signal, or barrier disruption.[71,84] Decreased immune cell trafficking can contribute to immunosuppression because immune cells would not be localized to destroy the pathogen and/or initiate adaptive responses. On the other hand, immune cell trafficking can contribute to chronic inflammation or autoimmune disease if increased numbers of immune cells are recruited to a site of insult or immune cells are inappropriately recruited to nontarget tissues, respectively. Indeed, there are immunomodulatory therapeutics that were purposefully designed to alter cell trafficking. Natalizumab is a monoclonal antibody that targets the α4 chain of the integrin, very late antigen (VLA) 4, on T cells, thereby disrupting its interaction with vascular cellular adhesion molecule (VCAM) 1 on endothelial cells.[114] The disruption of the VLA4–VCAM1 interaction prevents lymphocytes, including autoreactive lymphocytes, from penetrating the blood–brain barrier and infiltrating the central nervous system (CNS).[114] Imiquimod, a TLR 7 agonist, can produce intense local inflammatory reactions as a result of its ability to enhance cell trafficking. In mice treated with melanoma antigen peptide–pulsed DCs as a cancer immunotherapy, topical imiquimod enhanced the trafficking of the DCs into draining lymph nodes.[115] Imiquimod-treated mice also exhibited increased infiltration of immune cells into the tumor microenvironment.[115]

There are also potential immunotoxicants that exhibit immune suppression via suppression of cell trafficking. For instance, cannabinoid receptor 2 (CB2) ligands such as JWH-133 exhibited reduced ability to adhere to LPS-activated endothelial cells.[116] JWH-133 suppression of leukocyte adhesion was due in part to suppression of conformationally correct forms of integrins β1 and β2.[116]

KC9: Alters Cell Death Processes

Programmed cell death is important for a balanced immune system. However, unregulated death of immune cells may cause immunosuppression, resulting in the development of cancer, the inability to fight infections, and autoimmunity. Apoptosis, autophagy, and pyroptosis are all forms of programmed cell death that, if unregulated, may result in immunotoxicity.[117] Apoptosis is characterized by cell shrinkage, nuclear condensation, changes in the cell membrane and mitochondria, DNA fragmentation, and protein degradation by caspases. It plays a critical role in the

development and homeostasis of the immune system, including thymic selection, deletion of autoreactive cells, and maintaining the appropriate number of leukocytes in the periphery.[118] Because of its important role in homeostasis, defects in normal immune cell apoptosis can lead to disease. For example, glucocorticoids and CsA, used as immunosuppressive and anti-inflammatory agents, can induce dose-dependent apoptosis of thymocytes.[119] In addition, T-2 toxin and high-dose exposure to TCDD have been shown to enhance thymocyte apoptosis in rodents and other species.[120,121] Increased apoptosis can also reduce the number of lymphocytes, leading to severe infections and emergence of neoplasia that has been demonstrated through HIV depletion of $CD4^+$ T cells.[122] In addition, autoimmunity can be a consequence of improper clonal selection and negative selection that can lead to increases in self-reactive T and B cells.[27]

Autophagy is another type of programmed cell death, in which cell homeostasis is maintained by eliminating damaged or aged cells, organelles, and cell waste, subsequently providing the building blocks and energy for new and/or remaining cells.[117] Under normal physiologic conditions, autophagy is maintained at a basal level to ensure the turnover of damaged components, maintain cellular homeostasis, and support cellular metabolism. However, nutrient deprivation, hypoxia, oxidative stress, infection, hormonal stimulation, DNA damage, and other exogenous stimulators can trigger autophagy and ultimately result in autophagic dysfunction and excessive autophagic cell death. Dysregulation of autophagy is thought to result in immunosuppression following exposure to heavy metals, such as cadmium,[123] and pesticides.[124] In addition, silica nanoparticles have been recently reported to induce increased cytotoxicity through autophagy and apoptosis in monocytes and macrophages.[125]

Pyroptosis is a form of programmed cell death that is uniquely dependent on caspase-1 activation and inflammation and which results in membrane osmotic lysis, cell disruption, and pro-inflammatory cytokine release.[117] Thus, in contrast to apoptosis and autophagy, in pyroptosis, the membrane bursts and cytosolic contents are released into the extracellular space. Caspase-4, -5, and -11, which are also expressed in non-monocytic cells, can induce pyroptosis upon recognition of intracellular lipopolysaccharide.[126] With the discovery of gasdermin D (GSDMD), a substrate of both caspase-1 and caspase-4/5/11, pyroptosis is considered a form of GSDMD-mediated programmed necrosis.[127]

Pyroptosis is triggered by various pathological and exogenous stimuli.[128] Regulation of macrophage pyroptosis has been shown to modulate excessive inflammation, providing new ideas for potential therapeutic approaches and mechanisms by which environmental agents perturb immune function.[129] In addition to increasing autophagy, heavy metals have also been shown to increase pyroptosis through inflammasome-mediated inflammation.[130] Crosstalk between the three types of programmed cell death has been reported,[117] but this is still an area of active investigation.

KC10: Breaks Down Immune Tolerance

Tolerance is accomplished through multiple mechanisms that allow the immune system to distinguish self from nonself (i.e., pathogens) and prevent the generation of an immune response to an organism's own cells and tissues.[27] T and B cells are normally educated in the thymus and bone marrow, respectively, so that they do not react against self-proteins (central tolerance).[131,132] However, there are T and B cells that weakly bind to self-proteins circulating in the blood. These cells are normally unresponsive (functional anergy) because of the factors that contribute to the regulation of the immune response and the maintenance of peripheral tolerance, including a lack of costimulatory signals and the production of inhibitory molecules, ignorance of tissue specific antigens, and active suppression by regulatory T cells.[27] When tolerance is broken, the immune system may react to self-proteins, leading to autoimmune disease. In addition, because the fetus is essentially an allograft in the uterus, immune tolerance is a critical factor in maintaining successful pregnancy.[133,134] Maternal antiphospholipid and anti-thyroid autoantibodies have been suggested as contributing factors in recurrent miscarriage.[135] Because of the female predominance for many autoimmune diseases, the effects of estrogen, synthetic hormones, and endocrine-disrupting chemicals on self-tolerance have been widely investigated.[136] These agents can act directly on T cells in the thymus or on thymic epithelial cells, modulating signal transduction pathways, DNA methylation, or transcriptional regulation to alter central tolerance.

Cell surface proteins such as CTLA-4 and PD1/PD-L1 serve as checkpoints in the regulation of the immune response, and genetic deficiencies in these molecules result in a spectrum of autoimmune disorders.[137] The increased use of monoclonal antibodies that block immunoregulatory molecules (e.g., checkpoint inhibitors such as nivolumab, pembrolizumab, and ipilimumab) as anticancer agents has led to immune-related adverse events, including autoimmune manifestations in 5%–15% of patients given that these compounds block inhibitory pathways, leading to unregulated T-cell activation and a breakdown in immune tolerance.[85,94,95]

Similarly, drugs such as procainamide and hydralazine mitigate immune tolerance through their effects on DNA methylation, resulting in the overexpression of cell surface molecules associated with the TCR (e.g., lymphocyte function-associated antigen 1/CD11a) and unrestricted T-cell activation, which may lead to the development of autoimmune syndromes that resemble systemic lupus erythematosus in susceptible individuals.[138,139]

In some instances, the production of antibodies against self occurs because the secondary or tertiary structure of a drug or protein-bound drug is similar to that of a self-protein.[138] Agents that damage cellular membranes and induce inflammation may enhance recognition of self-antigens and increase the production of costimulatory signals such as cytokines and other soluble mediators that perpetuate the immune response or augment the nonspecific production of antibodies, some of which may be autoreactive.[27,140] Carbon tetrachloride is a toxicant that induces autoimmune hepatitis in this manner, with cell damage leading to the recruitment of T and B cells and the increased production of pro-inflammatory cytokines, which results in self-recognition and an immune response against the liver enzyme cytochrome P450 2D6.[141]

Although environmental and occupational exposures to mercury have been associated with elevated levels of inflammatory markers and autoantibodies, there is insufficient epidemiologic evidence to establish a causative relationship between mercury exposure and autoimmune disease in humans.[142] In contrast, there is an extensive body of literature demonstrating that in rodent models, mercury can induce a loss of self-tolerance, polyclonal activation of T and B cells, and an enhancement of inflammatory pathways, leading to the development of autoimmune disease.[142,143]

Breaking tolerance to self-antigens results in autoimmune responses, whereas breaking tolerance to food antigens results in food allergies. The development of oral tolerance during infancy serves an important function in preventing food allergies. Early life exposure to some chemicals, such as bisphenol A, has been shown to interrupt the formation of tolerance.[144,145] Few chemicals have been tested for the potential to modulate food tolerance, and there is a need for additional research on mechanisms by which exposures may break oral tolerance.

Examples of How Immunotoxic Agents May Produce Immune Dysfunction and Disease via Their KCs

The KCs described above are based on established properties of known immunotoxicants. Some compounds may display multiple KCs and produce a range of immunotoxic effects that occur sequentially or through multiple independent pathways. Two examples provided in the descriptions of the KCs illustrate that well-established immunotoxic chemicals exhibit multiple KCs responsible for their adverse clinical effects: the immunosuppressive drug CsA and the AhR ligand TCDD. CsA demonstrates 6 of the 10 KCs (KCs 3–7 and 9) through inhibition of calcineurin A and the modification of transcription and cell death (Figure 2). TCDD exhibits 9 of the 10 KCs (KCs 2–10), and—via the AhR—causes effects in multiple cell types (Figure 3).[101–104]

Relevance of the KCs to Developmental Immunotoxicity

In developing the KCs of immunotoxicants, we largely considered effects of direct action on the mature immune system because this has been the most active area of investigation for both therapeutics and environmental agents. There may be particularly critical windows during development of the immune system that are more sensitive to immunotoxic insults, such as during pluripotent stem cell development or organogenesis, and in which some KCs become more important.[146] Developmental effects have been reported at lower doses, relative to adult exposure, and some consequences of developmental exposure persist until later in life[67,146] or span generations.[147] In addition, exposure to xenobiotics during embryofetal and/or peri/postnatal development may affect the immune system, yet the consequences are not obvious until later in life.[67,148,149] The KCs of immunotoxicants apply equally well to the developing immune system, although some underlying initiating mechanisms may be distinct from those that cause toxicity to the fully mature immune system. For example, evidence in mice shows that prenatal exposures can persistently disrupt DNA methylation, altering cell differentiation (KC5) and proliferation (KC4) in response to an immune challenge later in life.[148] There is currently not compelling evidence that changes in DNA methylation underlie immune modulation occurring with environmental exposures outside the developmental period. The KCs of immunotoxicants may also influence the interplay between the immune system of the mother and the conceptus, placental barrier function, and immunity, although few experimental studies have yet to directly interrogate this.

Assays to Evaluate the 10 KCs of Immunotoxicants

The assessment of the immunotoxic potential of a xenobiotic may include a variety of approaches and combination of fit-for-purpose assays. Over the years, salient publications and/or regulatory guidance documents outlined testing approaches, mostly using laboratory animals, to assess a combination of innate, and cell-mediated and humoral-mediated immune function[16,150–154] All of these approaches rely on interrogating end points that are more or less proximal to the 10 KCs described herein, and we suggest that future approaches should consider all of these KCs, where practicable, to ensure a comprehensive and precise evaluation of immunotoxicity. These KCs will also support alternative approaches to evaluate immunotoxicity. Although testing strategies in laboratory animals are well established, there are no test guidelines to detect chemical-induced immunotoxicity *in vitro* and no consensus on which assays should be included.[155] Thus, there is a need for novel *in vitro* approaches (particularly using human cells for translational relevance) to evaluate immunotoxicity. No single test will be able to assess all of the potential adverse effects of exposures on the immune system. Therefore, it is likely that a larger set of assays, covering the full scope of the KCs of immunotoxicants, will be needed. Presently, some assays interrogate very specifically the interaction of a xenobiotic with an immune-related target (e.g., the ability of a molecule to haptenize a naturally presented HLA-DR peptide, an example of KC2), whereas others interrogate the general health of an animal for evidence of immune disturbance (e.g., observation of clinical or anatomic pathology). In practice, the integration of multiple assays should be used to interrogate the potential for a xenobiotic to cause immunosuppression, immunostimulation, hypersensitivity, autoimmunity, or other deleterious effects on the immune system. To be considered immunotoxic, an agent need not exhibit all 10 KCs. For example, immunosuppression may involve several of the proposed KCs. Xenobiotics that interfere with proliferation, differentiation, and/or programmed death processes of immune cells (KC4, KC5, KC9) could result in immunosuppression. These KCs can be measured in a variety of *in vitro* and *in vivo* test systems, including hematopoietic stem cell differentiation assays (e.g., colony-forming units assays),[156] white blood cell counts, microscopic examination of lymphoid tissues, and enumeration by immunophenotyping of B cells, $CD4^+$ and $CD8^+$ T cells, NK cells, and other subpopulations of leukocytes.[152] The proliferative response of lymphocytes to mitogenic stimuli can be measured *in vitro*.[63,157] Alteration of differentiation and/or proliferation can be the result of altered signaling (KC3), which can be assessed biochemically using pathway-specific targeted approaches. Immunosuppression may also be the result of altered function for specific cell types (KC7), and multiple assays are available to specifically interrogate phagocytosis, NK cell function, CTL function, and T cell-dependent or -independent antibody responses.[111,152] Such altered immunity may also result from perturbation of cell trafficking (KC8) best assessed by a combination of immunophenotyping and anatomic pathology end points.[158] Immunostimulation (as a consequence of KC6) may be assessed by a combination of cytokine release related assays (*in vitro* in human cells or *in vivo*) and clinical and anatomic pathology end points included in animal toxicology studies. Immunostimulation associated with increased T-cell activity may also lead to a break in tolerance mechanisms (KC10) best assessed via anatomic pathology end points or circulating autoantibodies.[159] The potential for a xenobiotic to haptenize a protein (KC1, KC2 for MHC-associated peptides) can be assessed using *in silico* or *in chemico* methods (direct-peptide reactivity assays[160]), *in vitro* reactivity assays (KeratinoSens[161]; human Cell Line Activation test[162]), as well as *in vitro* cellular assays measuring the ability of such haptenized proteins/peptides to stimulate an immune response (lymphocyte transformation test[163]). *In vivo* methods are also available to measure either the initial lymphoproliferation caused by haptenized proteins (local lymph node assay[164]) or the elicitation of hypersensitivity caused by hapten/antigen challenge (guinea pig or human skin sensitization assays). There is recognition that despite the availability of these approaches, the prediction of xenobiotic-induced hypersensitivity reactions (in particular, systemic hypersensitivity) remains challenging.

Discussion

There are several ways in which the KCs of immunotoxicants identified here could be used to enhance current practices in the clinic, pharmaceutical development, biomedical research, and assessment of health risks from exposure to environmental agents and consumer products. Substances acting through each of the KCs may contribute to clinical disease, with KC1 and KC2 being the predominant mechanism for increased hypersensitivity, and KC10 contributing mainly to increased autoimmunity, inflammation, and recurring miscarriage (Figure 4). KC3–9 can, singly or

in combination, contribute to immune suppression or immune dysregulation and contribute to increased risk of multiple diseases (Figure 4). It is the authors' opinion that awareness of the KCs of immunotoxicants can improve and accelerate understanding of the pathogenesis of these disease processes. Further, we opine that knowledge of the properties that cause small and large molecules to be immunotoxic will also help pharmaceutical companies and others to develop medicines that have a more favorable benefit–risk profile and can inform the replacement of potentially immunotoxic consumer products with safer components.

Regulatory agencies and authoritative bodies throughout the world conduct human health risk assessments by considering the evidence through various approaches that exposure to a given substance is associated with a health effect. These agencies typically attempt to use the most sensitive systems to characterize harmful exposures and identify safe exposure levels. Data permitting, they consider effects on any and all organ systems when determining whether a drug or an exogenous exposure poses a risk. Conclusions on the potential risk for an adverse immune effect or altered immune function are based on the integration of available studies in humans, experimental animals, and mechanistic data, typically using a systematic review approach.[2,165] The 10 KCs described above provide a framework for evaluating the mechanistic information that will help to better identify and understand hazards and risks to the human immune system. Mechanistic data are critical to this evidence integration for both hazard and dose–response assessment. They can inform the early events in the pathogenesis process, the relevance of apical end points in human population-based and animal studies, and the selection of critical studies for dose–response analysis, thereby increasing confidence in the overall health effects conclusions. Although many studies that have examined immunotoxicity have not directly compared different doses, it is likely that the dose–response relationship may not be the same across metrics of immune responses (i.e., across all of the KCs of immunotoxicity). For example, even an immunotoxic chemical as potent as TCDD shows variation in the dose that leads to a specific functional change. Higher doses of TCDD [$\geq$15 μg/kg body weight (BW) to mice] induce thymic atrophy and also perturb T- and B-cell responses to a range of antigens.[57,166,167] However, exposure to $\leq$10 μg/kg BW no longer affects thymic atrophy but still represses T- and B-cell proliferation and differentiation.[56,168,169] This suggests that, at least for AhR ligands, KC9 (cell death processes) may have a different dose–response relationship than KC4 (proliferation) or KC7 (effector function).

It should also be noted that we do not equate immunotoxic potential with the number of KCs altered, although many of the KCs are interrelated. In our opinion, the likelihood of an agent exhibiting KC4 (alters immune cell proliferation) in the absence of exhibiting any other KCs (i.e., KC3, alters immune cell signaling or KC6, alters immune cell–cell communication) is low. Varying dose–response relationships are another reason why we do not equate immunotoxic potential with number of KCs altered. If an agent "only" exhibits KC4 (proliferation) at very low doses as compared with another agent exhibiting several KCs at higher doses, the immunotoxic potential for the agent exhibiting immune cell proliferation is higher than the agent affecting several other KCs.

Further, it is the authors' opinion that mechanistic studies are increasingly important for pharmaceutical and toxicology research, and the KCs of immunotoxicants will help to contextualize results across different levels of biological organization. We also opine that the KCs can also be used to develop targeted literature searches and screening strategies to identify and assess relevant data on immune mechanisms and as an organizational framework to support synthesis and interpretation of evidence from human, experimental animal, and mechanistic studies in a systematic manner.[2,165]

Tremendous progress has been made in developing methods to assess immunotoxicity in the past decades. It is the expert opinion of the authors that the next generation of immunotoxicology assessment will require the adaptation and integration of novel approaches and strategies to better predict potential hazards, further reduce the use of animals, and expand the repertoire of immune end points and functions encompassed in immunotoxicity testing. Among these, we predict that new approach methodologies anchored in the identified KCs of immunotoxicants, along with a combination of complementary information, offer an opportunity to predict, identify, and ameliorate hazards that xenobiotics may pose to the immune system.

The KC approach is highly applicable to immunotoxicants and, similar to the KCs developed for other forms of toxicity,[1,2,8–12] provides a framework to evaluate existing data for risk assessment and regulatory activities, identify knowledge gaps, and facilitate the design of novel methods to evaluate the impact of xenobiotics on immunity.[1] We noted that five of the KCs associated with immunotoxicity reflect shared mechanisms that can be found in several target organs and tissues (KCs 3, 4, 5, 6, and 9), such as the liver[11] or the reproductive system,[9] whereas the other five KCs reflect the unique nature of interactions with components of the immune system (KCs 1, 2, 7, 8, and 10). A mechanistic understanding of how substances cause immunotoxicity earlier in the drug discovery or risk assessment process will advance the development of safer products using systematic and defined testing strategies for the comprehensive evaluation of immunotoxicity in humans, in our expert opinion. Although it has been examined in a number of analyses,[153,170] the relationship between suppression of functional immune measures and clinical disease remains uncertain at the lower end of the curve. As we continue to assess functional immune responses in the human population (e.g., antibody titers and vaccine efficacy) we may be able to better address whether there is a threshold where mild-to-moderate immunosuppression translates into clinical disease.[170,171]

We conclude that the use of these KCs will improve efforts to identify agents that cause alterations in immune function, to develop better testing and biomarker approaches to evaluate immunotoxicity, and to enable a more comprehensive and mechanistic understanding of adverse effects on the immune system. We recommend that such KCs be leveraged when testing guidelines are developed or revised by regulatory bodies.

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