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12.11 Alterations in Blood Components

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Glossary

ACD Anemia of chronic disease
AIHA/AHA Autoimmune hemolytic anemia
AML Acute myeloid leukemia
ATP Adenosine triphosphate
BFU-E Burst-forming unit-erythroid
BFU-Mk Burst-forming unit-megakaryocyte
C3a Complement 3a

C5a Complement 5a
CALR Calreticulin encoding gene
CARPA Complement activation-related pseudoallergy
CD Cluster of differentiation
CFU-E Colony-forming unit-erythroid
CFU-GM Colony-forming unit-granulocyte macrophage
CFU-Mk Colony-forming unit-megakaryocyte
CHCM Corpuscular mean hemoglobin concentration
COPD Chronic obstructive pulmonary disease
CUBAM Ileal cubilin and amnionless complex receptor
CXCL12 C-X-C motif chemokine ligand 12
DAT Direct antiglobulin test
DIC Disseminated intravascular coagulation
EGLN1 Egl-9 family hypoxia-inducible factor 1 gene
EPO Erythropoietin
FAD Flavin adenine dinucleotide
Fe^{2+} Ferrous iron
Fe^{3+} Ferric iron
FeLV Feline leukemia virus
FIV Feline immunodeficiency virus
G6PD Glucose-6-phosphate dehydrogenase
G-CSF Granulocyte colony-stimulating factor
GATA2 GATA binding protein 2
GM-CSF Granulocyte-macrophage colony-stimulating factor
HIF-1 Hypoxia-inducible factor 1
HIV Human immunodeficiency virus
HUS Hemolytic uremic syndrome
IL Interleukin
IMHA Immune-mediated hemolytic anemia
IMT Immune-mediated thrombocytopenia
INFγ Interferon gamma
ITP immune-mediated thrombocytopenic purpura
JAK Janus kinase
LNK Lymphocyte-specific adaptor protein
M-CSF Macrophage colony-stimulating factor
MCHC Mean corpuscular hemoglobin concentration
MCV Mean corpuscular volume
MPL Thrombopoietin receptor encoding gene
MPV Mean platelet volume
NK Natural killer
nRBC Nucleated red blood cell
NSAID Nonsteroidal antiinflammatory drug
***P*450** Cytochrome *P*450
PARR Antigen receptor rearrangement
PCR Polymerase chain reaction
PF4 Platelet factor 4
PFCP Primary familial and congenital polycythemia
PFK Phosphofructokinase
PIMA Precursor-targeted immune-mediated anemia
PK Pyruvate kinase
PRCA Pure red cell aplasia
RAG Recombination activating gene
RANTES Regulated on activation, normal T-cell expressed and secreted
SCF Stem cell factor

SCID Severe combined immunodeficiency
SIV Simian immunodeficiency virus
SLE Systemic lupus erythematosus
TGFα Transforming growth factor alpha
TGFβ Transforming growth factor beta
T_H1 T-helper cell type 1
T_H2 T-helper cell type 2
TNFα Tissue necrosis factor alpha
TPO Thrombopoietin
TTP Thrombotic thrombocytopenic purpura
VHL von Hippel-Lindau tumor suppressor

12.11.1 Introduction

An increasing number of xenobiotics are associated with alterations in the hematopoietic system. These alterations encompass a variety of direct and indirect effects, many of which are associated with the intended pharmacology of the compound but may also be related to off-target effects. Alterations in the hematopoietic system are commonly reflected by changes in the peripheral blood components. Blood components include leukocytes, erythrocytes, and platelets. Leukocyte subtypes in humans and common laboratory species include neutrophil, lymphocyte, monocyte, eosinophil, and basophil counts. The erythrocyte component includes both total erythrocyte and reticulocyte counts. The complete blood count (CBC) is the standard method for analyzing blood leukocyte, erythrocyte, and platelet components. Absolute concentrations (cells μL^{-1}), conventionally referred to as absolute counts in nonclinical toxicity studies, should be used to evaluate and interpret changes in blood components because relative percentages do not account for the total numbers of cells present and may be misleading.

Interpretation of xenobiotic-related alterations in blood components should be made with the consideration of the inherent biological variation in CBC endpoints observed in common laboratory species, and an understanding of the various mechanisms underlying increased or decreased blood component counts. There is considerable overlap in the mechanisms of blood cell alterations in both naturally occurring and xenobiotic-induced changes. The focus of this article is to review more commonly observed alterations in blood leukocyte, erythrocyte, and platelet components.

12.11.2 Leukocytes

Common patterns of alterations in blood leukocyte components are summarized in Table 1.

12.11.2.1 Neutrophils

The production of neutrophils, or granulopoiesis, occurs predominantly in the bone marrow, although extramedullary hematopoiesis may contribute, particularly in rats and mice, and if increased tissue demand for granulocytes or bone marrow disease is present.

Table 1 Classic patterns of alterations in blood leukocyte components

			Inflammatory patterns			
	Epinephrine	*Glucocorticoid*	*Overwhelming*	*Acute*	*Chronic*	*Bone marrow suppression*
Segmented neutrophils	↑	↑	↓ to ↓↓↓[c]	↑ to ↑↑↑[c]	↑ to ↑↑↑	↓ to ↓↓↓
Band neutrophils	-	-[a]	- to ↑↑[c]	↑ to ↑↑[c]	- to ↑	-
Lymphocytes	↑	↓	↓ to ↓↓	↓ to ↓↓	- to ↑↑[d]	↓ to ↓↓
Monocytes	- to ↑	- to ↑	- to ↓	- to ↑↑	- to ↑↑	↓ to ↓↓↓
Eosinophils	- to ↓	↓[b]	- to ↓[b]	↓[b] to ↑	- to ↑	↓[b]
Basophils	-	- to ↓[b]	- to ↓[b]	↓[b] to ↑	↓[b] to ↑	↓[b]

[a]May be slightly increased in some cases.
[b]Due to low eosinophil and basophil counts in health, decreases may be difficult to identify.
[c]Morphologic changes indicative of rapid neutropoiesis (Döhle bodies, cytoplasmic vacuolation, and cytoplasmic basophilia) may be observed.
[d]A proportion of blood lymphocytes may appear reactive.
Patterns described in this table indicate classic or expected changes in blood leukocyte counts due to these relatively common processes. However, duration of these processes, variations in the underlying causes of these conditions, and superimposition of multiple processes may result in differences between expected patterns of leukocyte changes and actual changes observed in an individual.
-, no apparent change; ↑, mild increase; ↑↑, moderate increase; ↑↑↑, marked increase; ↓, mild decrease; ↓↓, moderate decrease; ↓↓↓, marked decrease.

Stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-6 promotes the differentiation of common myeloid progenitor cells to granulocyte/monocyte progenitor cells, and subsequent stimulation with granulocyte colony-stimulating factor (G-CSF) promotes differentiation, proliferation, and maturation of granulocytes from myeloblasts to mature segmented neutrophils (Radin and Wellman, 2010). Granulocyte pools in the bone marrow can be divided into proliferating (mitotic) and maturing (postmitotic) pools. The proliferating pool encompasses the most immature myeloid precursors: myeloblasts, promyelocytes, and myelocytes. The maturing pool includes the later stages of metamyelocytes, band neutrophils, and mature segmented neutrophils. The mature segmented neutrophil population within the maturing pool is also considered the bone marrow storage pool, and acts as a reserve store of neutrophils that replenish blood stores following normal turnover and can be quickly mobilized to the blood in times of increased tissue demand.

In health, mammalian blood neutrophils are mature, segmented neutrophils. They are distributed in two pools within the blood vessels, the circulating pool and the marginating pool. Circulating pool neutrophils flow freely through the vessel lumen and are sampled during blood collection. Marginating pool neutrophils are in contact with endothelial cells and temporarily adhere to or roll along the vessel wall. Neutrophils may shift between the circulating and marginating pools. The lifespan of a neutrophil in circulation is short (approximately 7 h half-life in blood), and ultimately neutrophils migrate into tissues where they survive for a few days (Smith, 2016). Neutrophils are part of the innate immune system and primarily function as phagocytes to remove and destroy invading pathogens, but also can secrete proinflammatory and chemotactic mediators to enhance responses of both the innate and adaptive immune systems.

In blood, neutrophils are typically the most numerous leukocyte in humans, rhesus monkeys, African green monkeys, and dogs. However, many New World monkeys tend to have lymphocyte counts greater than neutrophil counts (Provencher Bolliger et al., 2010), and cynomolgus monkeys tend to initially have predominantly lymphocytes in circulation when they are young but shift to predominantly neutrophils with maturity (Sugimoto et al., 1986). Neutrophil counts are lower than lymphocyte counts in rats and mice. Blood neutrophil counts may increase or decrease depending on the stimulus or insult.

Clinically, increases in neutrophil counts above expected values in health (usually conveyed in a reference interval) may be referred to as neutrophilia or granulocytosis, while decreases in neutrophil counts may be called neutropenia, granulocytopenia, or, if severe, agranulocytosis. However, in nonclinical toxicology studies, the convention is to not use such clinical terminology to describe alterations caused by a test article. Referring to changes as increases or decreases in neutrophil counts is the standard for nonclinical toxicology studies, and this terminology is used throughout this article. However, clinical terms are also provided for reference.

12.11.2.1.1 Increases in neutrophil counts (neutrophilia, granulocytosis)

12.11.2.1.1.1 Catecholamine-induced

Acute, transient increases (typically <1 h) in neutrophil counts that are mild (typically increases of twofold or less) can occur in response to endogenous catecholamine stimulation (e.g., epinephrine), and are accompanied by increases in other blood leukocyte counts (physiologic leukocytosis). Increases in circulating catecholamines are commonly associated with excitement, fear, or exercise, and at times are referred to as acute stress. Increases in neutrophil counts secondary to catecholamine stimulation are mostly attributable to a shift in neutrophils from the marginating pool to the circulating pool, resulting in increased numbers of freely flowing neutrophils captured during blood sampling, and may occur through decreased expression of leukocyte adhesion molecules and potentially from increased blood flow and shear forces (Foster et al., 1986; Benschop et al., 1996; Stockham and Scott, 2008a; Everds et al., 2013a). Splenic contraction resulting in redistribution of sequestered neutrophils to circulation also contributes. Mechanisms by which exogenous catecholamines and adrenergic agents cause increases in blood neutrophil counts are the same as those described for endogenous catecholamine stimulation.

12.11.2.1.1.2 Glucocorticoid-induced

The pattern of glucocorticoid-related alterations in leukocyte counts is commonly referred to as a stress leukogram. The predominant endogenous steroids are cortisone in most species or corticosterone in the rat. Classically, endogenous steroids cause increases in neutrophil counts that are accompanied by decreases in lymphocyte counts, increases in monocyte counts, and decreases in eosinophil counts. However, these effects may be variable depending on the species, and increases in neutrophil counts may be less prominent or absent in rats and mice. Interpretation of a stress or endogenous glucocorticoid effect in a nonclinical toxicity study should be made conservatively using a weight of evidence approach. It should also be noted that hyperadrenocorticism (Cushing syndrome or hypercortisolism) is associated with excess production of endogenous glucocorticoids, and will also cause increases in blood neutrophil counts through glucocorticoid-mediated mechanisms.

Glucocorticoids increase blood neutrophil counts through several mechanisms. They downregulate neutrophil expression of adhesion molecules, leading to both a shift from marginating to circulating pools, and by prolongation of neutrophil lifespan in circulation due to decreased tissue emigration (Stockham and Scott, 2008a). Neutrophil half-life may also be prolonged due to decreased apoptosis (Cox, 1995). These increases in neutrophil lifespan may be associated with hypersegmentation of neutrophil nuclei that can be observed during blood smear evaluation. Glucocorticoids also increase the release of segmented and occasionally band neutrophils from the bone marrow storage pool, although this mechanism contributes to the neutrophilia to a lesser degree than shifting to the circulating pool (Stockham and Scott, 2008a; Nakagawa et al., 1998). Bone marrow expansion of all stages of myeloid cells may also be increased by glucocorticoid stimulation (Trottier et al., 2008). Administration of exogenous glucocorticoids, such as prednisolone and dexamethasone, will result in similar effects on blood leukocyte counts.

12.11.2.1.1.3 Inflammation

Inflammation is one of the most common causes of increased blood neutrophil counts. Inflammation is typically further characterized as acute or chronic, although these terms are often used inconsistently across disciplines and may refer to either the duration of the inflammation or the type of reaction to the inflammatory response. The latter definition is used here for alterations in blood leukocyte counts.

Acute inflammatory stimulation results from a tissue insult or invading pathogen that causes the release of proinflammatory mediators. Proinflammatory mediators in the stimulation of acute inflammatory neutrophilia include IL-1, IL-8, and TNFα (Dinarello, 2000; Kolackzowska and Kubes, 2013). These changes cause a rapid initial release from the bone marrow storage pool into circulation that generally occurs within 8 h. Increased neutrophil counts are observed when bone marrow release exceeds emigration of neutrophils to the site of the inflammatory insult. With persistence of the inflammatory stimulus, the bone marrow storage pool becomes depleted and more immature neutrophil stages are released into circulation.

The presence of immature neutrophils in blood is called a left shift. These immature neutrophils are most commonly band neutrophils, but a sufficient stimulus can result in the release of lower numbers of more immature stages, including metamyelocytes, myelocytes, or even earlier stages. A left shift may be categorized as degenerative, indicating a greater number of immature neutrophils than mature neutrophils, or regenerative, where mature neutrophils are present in greater numbers than the immature neutrophils. Degenerative left shifts are typically observed in the face of decreased or low-normal blood neutrophil counts when the tissue demand exceeds bone marrow neutrophil production, while regenerative left shifts tend to occur with increased blood neutrophil counts when bone marrow production is starting to meet the tissue demand.

Bone marrow neutrophil production increases as the inflammatory stimulus depletes the neutrophil storage pool and recruits immature cells into circulation. The process of proliferation and maturation from the myelocyte stage to release of mature segmented neutrophils from the bone marrow takes approximately 6 to 9 days in health, although transit time can be decreased to 2 to 4 days with an acute inflammatory stimulus (Harvey, 2012; Stockham and Scott, 2008a). Due to acceleration of granulopoiesis, morphologic changes may occur in the cytoplasm of neutrophils. These changes include Döhle bodies (remnants of rough endoplasmic reticulum that contain RNA), foamy cytoplasmic vacuolation, increased cytoplasmic basophilia, and evidence of asynchronous cytoplasmic and nuclear maturation (e.g., large neutrophils, lobes of immature nuclear chromatin with filamentous segmentation, or presence of primary granules in more mature stages). These changes are often referred to as "toxic" changes, but they are more accurately cellular morphologic alterations that are considered an indicator of rapid/accelerated neutrophil production.

Causes of acute inflammation are many and varied. Bacterial and fungal infections are commonly associated with increases in neutrophil counts. Tuberculosis has been associated with increased neutrophil counts in humans and in macaques commonly used in nonclinical toxicology studies (Lowe et al., 2013; Magden et al., 2015). In macaques commonly used in nonclinical toxicology studies, shigellosis may cause neutrophilia with or without a left shift even in the absence of clinical signs and diarrhea (Magden et al., 2015). Macaques may also have increases in neutrophil counts with enteric infection by Yersinia species and respiratory tract infection by *Streptococcus pneumoniae* or *Klebsiella pneumoniae* (Magden et al., 2015). Increases in neutrophil counts can also be observed as a direct response to viral infections or secondary to viral-induced tissue damage, tissue damage from trauma, or as a paraneoplastic effect.

Once the inflammatory stimulus has persisted long enough to result in granulocytic hyperplasia of the bone marrow and at least partially replenished the neutrophil storage pool, increases in blood neutrophil counts are characterized by a diminishing left shift and a switch to predominant mature segmented neutrophils in circulation. Also, if the insult is effectively being resolved by the inflammatory response, tissue demand for neutrophils may decrease, resulting in release of fewer immature stages and the appearance of a chronic inflammatory pattern. In nonclinical toxicology studies, certain procedure-related effects, such as long-term catheterization, may cause an inflammatory leukogram (Hall, 2013).

In some cases, a leukemoid response or extreme neutrophilia may occur. A leukemoid response is characterized by a persistent leukocytosis of $>50{,}000$ cells μL^{-1}, typically due to a marked neutrophilia with a left shift that remains orderly and may or may not have morphologic changes indicative of rapid granulopoiesis (Schultze, 2010; Sakka et al., 2006). Extreme neutrophilia typically has $>100{,}000$ cells μL^{-1} and evidence of a left shift. The terms leukemoid reaction and extreme neutrophilia are most appropriately applied retrospectively, after the possibility for hematopoietic neoplasia has been excluded. Differentiation of a leukemoid response or extreme neutrophilia from chronic myelogenous leukemia or chronic neutrophilic leukemia includes CBC, blood smear, and bone marrow evaluations in most species, and may also include leukocyte alkaline phosphatase activity, immunophenotyping, cytogenetic analysis (e.g., evaluation for bcr–abl translocation), serum G-CSF, and clonality evaluations in humans (Schultze, 2010; Sakka et al., 2006). Leukemoid reactions have been associated with carcinomas of various origins, including renal and pulmonary carcinomas, Hodgkin's lymphoma, melanoma, and sarcomas, and may be attributable to aberrant production of proinflammatory mediators by the neoplasm, such as G-CSF, GM-CSF, or IL-6 (Sakka et al., 2006). Leukemoid reactions have also been reported in F334/N rats affected by large granular cell leukemia (Car et al., 2006). However, leukemoid reactions may also be associated with infectious processes, including disseminated tuberculosis, *Clostridium difficile* colitis, severe shigellosis (Sakka et al., 2006), chronic localized suppurative lesions such as pyometra, pleuritis, and internal abscesses (Schultze, 2010; Stockham and Scott, 2008a). Leukemoid reactions may also be seen secondary to severe hemorrhage or immune-mediated hemolytic anemia (Schultze, 2010; Sakka et al., 2006).

12.11.2.1.1.4 Inherited leukocyte adhesion deficiencies

Increases in neutrophil counts associated with deficiencies in leukocyte adhesion molecules may manifest as a leukemoid response or extreme neutrophilia. Adhesion molecules expressed on neutrophils are responsible for neutrophil margination, rolling along vessel walls, and emigration into tissues. L-selectin (CD62L) mediates low-affinity initial binding of leukocyte to endothelial cells, while integrins, including CD11b/CD18 (Mac-1), mediate firm adhesion to endothelial cells and ligands in the extracellular matrix (Muller, 2012). Neutrophils constitutively express CD11b/CD18. A deficiency of this integrin (leukocyte adhesion deficiency [LAD] type 1) results in the failure of neutrophils to emigrate to tissues, and may result in severe, recurrent bacterial infections (Arnaout, 1990). LAD type 2 is due to an inherited disorder of fucose metabolism, resulting in the lack of selectin ligands expressed on neutrophils and therefore results in immunodeficiency from a failure of selectin-mediated neutrophil rolling along vessel walls (Marquardt et al., 1999). Leukocyte adhesion deficiencies have been reported in humans, dogs, mice, and Holstein cattle (Arnaout, 1990; Marquardt et al., 1999; Gu et al., 2004).

12.11.2.1.1.5 Neoplasia

Neoplasms involving hematopoietic cells naturally occur with relatively low frequency. In general, such neoplastic processes may be observed as background findings in rats and mice during longer toxicity studies (e.g., carcinogenicity studies), but are uncommon in nonrodent species during toxicity studies (Smith et al, 2002).

Increases in neutrophil counts may be observed as part of the neoplastic processes of chronic myeloid leukemia (CML) or chronic neutrophilic leukemia (CNL). Leukocytosis in CML is $\geq$25,000 cells μL^{-1} with increases in all stages of neutrophilic myeloid cells in blood, which are typically accompanied by increases in monocyte counts, eosinophil counts, basophil counts, platelet counts, and possibly increases in nucleated erythroid precursors (Sawyers, 1999). In contrast to a leukemoid response, the left shift associated with CML tends to be less orderly, with greater numbers of earlier stages of myeloid cells in circulation along with the band and segmented neutrophils. Morphologic features indicative of dysplasia may be seen, and cytoplasmic features indicative of rapid granulopoiesis may be observed in neutrophils associated with CML. Bone marrow findings include hypercellularity with increased myeloid to erythroid ratios where myeloblasts and promyelocytes make up $<$10% of all cells (Sawyer, 1999). Most human cases of chronic myeloid leukemia have been associated with a translocation of chromosomes 9 and 22 (Philadelphia chromosome) resulting in the constitutively active BCR–ABL tyrosine kinase, an oncogene that induces leukemias (Sawyers, 1999). A comparable bcr–abl translocation has been observed in dogs (Breen and Modiano, 2008; Cruz Cardona et al., 2011; Culver et al., 2013), and transgenic and knock-in mouse models for bcr–abl translocation-induced CML have been utilized in research (Ren, 2005).

Chronic neutrophilic leukemia is also characterized by blood leukocyte counts $\geq$25,000 cells μL^{-1} that are composed of $>$80% segmented and band neutrophils (Uppal and Gong, 2015). These neutrophils may or may not have cytoplasmic changes indicative of rapid neutropoiesis. Unlike CML, the increases in neutrophil counts associated with CNL are typically not associated with morphologic features of dysplasia, the presence of earlier stages of myeloid cells (i.e. myeloblasts, promyelocytes, myelocytes, and metamyelocytes), increases in monocyte, eosinophil, or basophil counts, or increases in nucleated erythroid precursors (Uppal and Gong, 2015). Bone marrow changes associated with CNL include hypercellularity with increased myeloid to erythroid ratios, where the myelocyte through band neutrophil stages are increased without apparent increases in myeloblasts or promyelocytes (Uppal and Gong, 2015). As such, differentiation of CNL from leukemoid reactions or extreme neutrophilia may be difficult, and affected patients should be carefully evaluated to exclude causes of neutrophilia not associated with hematopoietic malignancy.

12.11.2.1.1.6 Xenobiotic-induced

There are numerous xenobiotics that can increase blood neutrophil counts, typically through similar mechanisms as described for naturally occurring increases in neutrophil counts. Several examples of these compounds are described later.

Administration of exogenous catecholamines, such as epinephrine, or some adrenergic agonists has similar effects as those mediated by endogenous catecholamines. Shifting of neutrophils from the marginating to the circulating pools is responsible for the increases in blood neutrophil counts, potentially from altered adhesion molecule expression or hemodynamic forces. Leukocyte subtypes have different receptors modulating their adrenergic effects, and neutrophils appear to be primarily affected by α-adrenergic receptors (Benschop et al., 1996).

Exogenous glucocorticoids, such as prednisolone, dexamethasone, and betamethasone, will mediate increases in neutrophil counts through similar mechanisms as endogenous glucocorticoids. In brief, these increases in neutrophil counts are due to shifting of neutrophils from the marginating to circulating pool, release of neutrophils from the bone marrow storage pool, and expansion of granulopoiesis in the bone marrow. The dose and duration of the glucocorticoid therapy may alter the proportional contribution of each of these effects to the increases in neutrophil counts.

Heparin-induced increases in neutrophil counts are reported in a small percentage of patients receiving heparin therapy, and the mechanisms are likely multifactorial. Heparin may cause shifting of neutrophils to the circulating pool from the marginating pool due to decreased expression of selectins (Zhang et al., 2012). Heparin-related release of CXCL12, a chemokine that is constitutively expressed by bone marrow stromal cells and plays a role in bone marrow neutrophil retention and leukocyte trafficking, may alter bone marrow and circulating CXCL12 gradients and promote release of neutrophils from the bone marrow storage pool, contributing to the increases in circulating neutrophil counts (Zhang et al., 2012).

Administration of G-CSF or GM-CSF may be used as a rescue therapy following chemotherapy that causes neutropenia and compromises the immune system's ability to respond to infectious agents. While the goal for treatment in chemotherapy patients is to increase blood neutrophil counts to normal levels and not to achieve increased neutrophil counts, repeated or high dose administration of G-CSF or GM-CSF-related compounds to healthy laboratory animals results in markedly increased neutrophil counts. Typically these changes are associated with a left shift and cytoplasmic changes indicative of rapid neutropoiesis. Dysplastic changes may also occur, most commonly recognized by the presence of unusually large neutrophils or hypersegmented neutrophils in circulation. Several xenobiotics appear to induce increases in neutrophil counts by stimulating increases in endogenous G-CSF or an inflammatory stimulus. Early in the course of treatment with clozapine, an antipsychotic agent, rats have been shown to have spikes in G-CSF with concomitant increases in bone marrow granulopoiesis and release of less mature neutrophils into circulation (Lobach and Uetrecht, 2014). However, other xenobiotics may directly cause an inflammatory stimulus and increase in endogenous proinflammatory stimuli, such as G-CSF and IL-1, which result in increases in neutrophil counts. For example, lithium, which has been used in treatment of bipolar disorder and in combination with antidepressants, also appears to cause increased neutrophil counts due to increased G-CSF (Focosi et al., 2009).

Xenobiotic-induced increases in neutrophil counts may be observed in conjunction with several cutaneous drug reactions that result in inflammatory stimuli. Acute generalized exanthematous pustulosis is a drug-related skin reaction that generally occurs within 2 days of drug administration but resolves spontaneously within 15 days (Roujeau, 2005). Increases in eosinophil counts may or may not be observed along with the increases in neutrophil counts. Xenobiotics classically associated with this syndrome include diltiazem and antibiotics such as aminopenicillins and pristinamycine (Roujeau, 2005). Acute febrile dermatosis (Sweet's syndrome) has also been associated with elevations in circulating neutrophil counts, and drug-induced forms of this condition have been variably linked to a variety of compounds, including GM-CSF, trimethoprim–sulfamethoxazole, minocycline, celecoxib, furosemide, and azathioprine (Rochet et al., 2013; Saavedra et al., 2006).

12.11.2.1.2 Decreases in neutrophil counts (neutropenia, granulocytopenia, agranulocytosis)

12.11.2.1.2.1 Overwhelming or severe acute inflammation

Although inflammation often causes increases in neutrophil counts, an overwhelming or severe acute inflammatory stimulus may result in decreases in neutrophil counts. Such an inflammatory stimulus, sometimes also referred to as peracute inflammation, is commonly observed with bacterial sepsis. Decreases in neutrophil counts are a result of emigration to tissues that exceed the bone marrow capacity to release neutrophils. Proinflammatory mediators and chemoattractants stimulate increased neutrophil margination, firm adhesion to endothelial cells, and emigration into tissue, which shortens neutrophil circulating half-lives and depletes the circulating neutrophil pool. As neutrophils are released from the bone marrow and the storage pool is depleted, recruitment of immature neutrophils into circulation results in a left shift in which the number of immature forms exceeds the number of mature segmented neutrophils (degenerative left shift). If the stimulus persists long enough, granulocytic hyperplasia of the bone marrow will occur and the numbers of circulating neutrophils will increase and shift toward greater numbers of mature than immature neutrophils.

12.11.2.1.2.2 Endotoxemia

Decreases in neutrophil counts may be observed as a result of Gram-negative bacterial infections that release endotoxin. Endotoxemia has been demonstrated to increase expression of the integrin CD11b/CD18 and decreased expression of L-selectin on neutrophils (Wagner and Roth, 1999), mediating a shift from circulating to marginating pools with firm adhesion to endothelial cells. There is evidence that this upregulation of CD18 contributes to neutrophil vascular sequestration in the lungs and liver, but there is also evidence for other mechanisms, such as cytoskeletal changes that alter neutrophil deformability, causing the initial vascular sequestration of neutrophils that is consistently observed with endotoxemia (Wagner and Roth, 1999). The decreases in neutrophil counts associated with endotoxemia tend to be rapid but transient, and detection of these decreases in neutrophil counts will depend on timing of the blood collection in relation to endotoxemic and other concurrent or subsequent proinflammatory stimuli.

12.11.2.1.2.3 Viral-induced

Decreases in neutrophil counts may be associated with viral infections, although viral-specific mechanisms vary. Decreased neutrophil counts associated with parvovirus likely represent both a primary effect due to infection causing death of rapidly dividing hematopoietic precursors in the bone marrow, as well as a secondary increase in tissue demand from disease and loss of integrity of the intestinal tract. Decreases in neutrophil counts associated with human immunodeficiency virus (HIV), infectious hepatitis, and infectious mononucleosis in people have also been associated with decreased or ineffective neutrophil production due to infection of hematopoietic precursors (Dale and Welte, 2016; Lima et al., 2006). With measles and dengue virus, decreases in neutrophil counts have been observed with endothelial cell alterations leading to increased adhesion of neutrophils and therefore a shift from the circulating to the marginating pool (Dale and Welte, 2016).

Decreases in blood neutrophil counts may also result from decreased neutrophil production, as in acquired viral immunodeficiency. Immunodeficiency syndromes with decreased neutrophil counts have been reported with infections by HIV in people, simian immunodeficiency virus (SIV) or simian betaretrovirus in monkeys, or feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) in cats (Levine et al., 2006; Israel and Plaisance, 1991; Magden et al., 2015; Gill et al., 2012; Gleich and Hartmann, 2009).

12.11.2.1.2.4 Immune-mediated

Primary or idiopathic immune-mediated destruction of mature neutrophils or neutrophil precursors will result in decreases in neutrophil counts, and has been reported in humans and most laboratory species. Antineutrophil antibodies are most commonly IgG and less commonly IgM class. Opsonization of neutrophil membranes may lead to leukoagglutination and neutrophil sequestration in sites including the spleen, liver, and lymph nodes, with phagocytosis by macrophages. Some antineutrophil antibodies may cause direct cytotoxicity through either complement-mediated or complement-independent mechanisms (Chickering and Prasse, 1981). In humans, immune-mediated neutrophil destruction due to antibody-dependent lymphocyte cytotoxicity has been reported (Logue et al., 1978).

Systemic lupus erythematosus (SLE) is an autoimmune condition that has been associated with decreases in neutrophil counts. These decreases in blood neutrophil counts appear to be relatively common with SLE, and are usually observed in conjunction with other manifestations of SLE, such as arthritis, skin lesions, or neurologic disorders (Stone, 2005). There is an association between decreases in neutrophil counts and the presence of autoantibodies in SLE (Hsieh et al., 2003; Budman and Steinberg, 1977), which lead to destruction or death of neutrophils. However, SLE-related anti-G-CSF autoantibodies have also been observed, causing decreased blood neutrophil counts due to decreased production rather than or in addition to direct neutrophil destruction or death (Hellmich et al., 2002).

Most cases of primary or idiopathic aplastic anemia result from underlying immune-mediated destruction of uncommitted or early lineage-committed hematopoietic stem cells (Young et al., 2006). Aplastic anemia is characterized by pancytopenia (marked decreases in all blood component counts) and hematopoietic cell hypocellularity in bone marrow. Although most commonly associated with immune-mediated destruction of hematopoietic precursors, marked depletion of bone marrow hematopoietic cells or aplastic anemia may also occur with severe nutritional deficiencies associated with anorexia nervosa (Abella et al., 2002) and 75% feed restriction in rats (Moriyama et al., 2008; Levin et al., 1993).

12.11.2.1.2.5 Cobalamin (B_{12}) and folate (B_9)

Deficiencies in cobalamin and folate cause disruption of DNA synthesis that results in megaloblastic hematopoiesis, commonly associated with megaloblastic anemia and occasionally with decreases in neutrophil counts and/or pancytopenia (Dale and Welte, 2016; Green, 2016). Cobalamin or folate deficiencies may be due to malnutrition, malabsorption from gastrointestinal disease or dysfunction, or genetic deficiencies of transport proteins, such as intrinsic factor, transcobalamin, or R-factor for cobalamin, receptors for intestinal absorption, such as the CUBAM receptor, or metabolizing enzymes, such as methylenetetrahydrofolate reductase for folate (Fowler, 1998; Whitehead, 2006; Montagle and Tauro, 1995; Green, 2016; Quadros, 2010). During megaloblastic hematopoiesis, asynchronous cytoplasmic and nuclear maturation may result in giant granulocytes with irregular nuclear chromatin patterns, such as giant metamyelocytes (Green, 2016), or hypersegmented neutrophils (Thompson et al., 1989). These conditions can often be managed with supplementation of the deficient vitamin. A differential for cobalamin or folate deficiency-induced decreases in blood neutrophil counts is copper deficiency, which can have a similar clinical manifestation (Lazarchick, 2012).

12.11.2.1.2.6 Myelophthisis, myelofibrosis, and myelonecrosis

Conditions that efface the bone marrow cavities or displace hematopoietic cells (myelophthisis) may result in decreased production of all hematopoietic cell lines, including neutrophils. Myeloproliferative syndromes (Tefferi and Vardiman, 2009), many leukemic diseases (Talcott et al., 1992; Lamy and Loughran, 1999), lymphoproliferative neoplasia (Schultze, 2010), or metastatic carcinoma (Makoni and Laber, 2004) can cause sufficient bone marrow overcrowding to decrease the production of neutrophils. Fungal infections resulting in granulomatous inflammation of the bone marrow, such as can be observed with disseminated histoplasmosis, may also result in decreased neutrophil production. Disruption of the bone marrow cavities and hematopoietic stem cells from myelofibrosis or myelonecrosis may also be associated with decreased blood neutrophil counts (Stockham and Scott, 2008a).

12.11.2.1.2.7 Xenobiotic-induced

Xenobiotic-induced decreases in neutrophil counts may be caused by immune-mediated or nonimmune-mediated mechanisms. The incidences of these events tend to be low with most implicated xenobiotics, with the exception of chemotherapeutic agents. Examples of both immune-mediated and nonimmune-mediated mechanisms are described later.

Similar to primary immune-mediated decreases in neutrophil counts, xenobiotic-induced immune-mediated decreases in neutrophil counts are commonly associated with IgG or IgM antineutrophil antibodies, although some drugs may induce both (Salama et al., 1989). Decreases in neutrophil counts by this mechanism tend to occur rapidly, within a few hours to 2 days after exposure (Schwartzberg, 2006). Some xenobiotics mediate their effects by acting as haptens, which induce an antibody response targeting an antigen formed by the xenobiotic–neutrophil combination. Drugs reported to act as haptens include penicillin, gold-based compounds, aminopyrine, and propylthiouracil (Salama et al., 1989; Murphy et al., 1985). These xenobiotics induce neutrophil destruction in a drug-dependent manner, and discontinuation of treatment generally results in resolution of blood neutrophil counts within 7 days (Schwartzberg, 2006). Some xenobiotics, including propylthiouracil and quinidine sulfate, cause the formation of immune complexes that can subsequently bind and destroy neutrophils, even if the xenobiotic is no longer present (Schwartzberg, 2006; Bhatt and Saleem, 2004; Eisner et al., 1977). In addition, some xenobiotics, such as propylthiouracil, may also cause the formation of antineutrophil antibodies resulting in complement-mediated neutrophil destruction (Akamizu et al., 2002).

Similar to idiopathic aplastic anemia, xenobiotic-induced aplastic anemia is most commonly associated with immune-mediated destruction of uncommitted or early hematopoietic stem cells, although direct cytotoxicity, such as with chemotherapeutic agents, may also lead to aplastic anemia. Several xenobiotics, including chloramphenicol, anticonvulsants such as phenytoin and carbamazepine, gold-based compounds, and phenylbutazone have been associated with aplastic anemia (Bloom and Brandt, 2008).

Numerous xenobiotics have also been linked to SLE, with decreases in blood neutrophil counts in some cases. In xenobiotic-induced SLE, decreased neutrophil counts reflect production of autoantibodies and subsequent neutrophil destruction, similar to nonxenobiotic-induced SLE. Xenobiotics associated with SLE include anticonvulsants such as phenothiazines, chlorpromazine, and valproate, antibiotics such as penicillin, streptomycin, tetracycline, griseofulvin, and sulphonamides, and miscellaneous xenobiotics such as captopril, phenylbutazone, and lovastatin (Stone, 2005; Mutasim and Adams, 2000).

In laboratory species used in nonclinical toxicology studies, transient xenobiotic-induced decreases in blood neutrophil counts have been associated with anaphylactoid reactions termed complement activation-related pseudoallergy (CARPA). These nonhypersensitive reactions are mediated instead by activation of the complement cascade, leading to the production of the anaphylatoxins C3a and C5a. CARPA typically occurs after the first dose of xenobiotic with decreasing severity or resolution following additional doses. Xenobiotics implicated in CARPA reactions include radiocontrast media, drug-carrying liposomes and lipid complexes, and some solvents with amphiphilic emulsifiers, such as Cremophor EL (Szebeni, 2005).

Nonimmune-mediated decreases in neutrophil counts may be observed with bone marrow suppression, which is associated with many xenobiotics, particularly chemotherapeutic agents. Chemotherapeutic-related decreases in neutrophil counts are often a result of direct cytotoxicity or suppressed proliferation of the rapidly dividing granulocytic precursors in the bone marrow. Examples of chemotherapeutic classes associated with decreased blood neutrophil counts include agents that cause direct DNA damage, such as platinum-containing agents (e.g., cisplatin, carboplatin) and classical alkylating agents (e.g., cyclophosphamide, melphalan, busulfan); mitotic spindle inhibitors (e.g., paclitaxel, docetaxel, vinblastine, vincristine); topoisomerase inhibitors (e.g., etoposide, doxorubicin); and antimetabolites (e.g., methotrexate, 6-mercaptopurine, 5-fluorouracil) (Bhatt and Saleem, 2004; Weiss, 2010; Wailoo et al., 2009). Decreases in neutrophil counts are expected with chemotherapeutic treatment, and can be easily monitored through serial CBCs. If severe enough to increase the risk of life-threatening infections clinically, treatment with empiric antibiotics or G-CSF-like compounds may be considered.

Suppression of granulopoiesis or direct granulocyte toxicity is also reported with some nonchemotherapeutic agents. Dose-dependent inhibition of colony-forming units of granulocytes and macrophages in the bone marrow has been reported with several anticonvulsant drugs, including valproic acid and carbamazepine, and beta-lactam antibiotics (Schwartzberg, 2006; Irvine et al., 1994; Watts et al., 1990; Neftel et al., 1985). Several other anticonvulsant drugs, including ethosuximide (Mintzer et al., 2009) and phenytoin (Sharafuddin et al., 1991), have been reported to cause direct toxic effects on granulocyte precursors. Antipsychotic agents, including clozapine and chlorpromazine, may also cause direct cytotoxic effects: neutrophil metabolism of clozapine has been demonstrated to form an unstable intracellular metabolite, nitrenium ion, which depletes glutathione and makes the neutrophils susceptible to oxidative damage and apoptosis (Williams et al., 2000); chlorpromazine may cause cytotoxicity through the inhibition of thymidine and uracil uptake by neutrophils (Pisciotta and Kaldahl, 1962). Thienopyridine inhibitors of platelet aggregation, including clopidogrel and ticlopidine, have also been associated with direct neutrophil toxicity resulting from mitochondrial toxicity by metabolites generated through myeloperoxidase metabolism of the parent compounds (Maseneni et al., 2012, 2013).

12.11.2.2 Lymphocytes

The production of lymphocytes, or lymphopoiesis, progresses from pluripotent hematopoietic stem cells that differentiate into common lymphoid progenitor cells, and further differentiate into B-cells, T-cells, and natural killer (NK) cells. B-cell development begins in the fetal liver and transitions to bone marrow postnatally, where the cells undergo proliferation and differentiation, followed by migration to the peripheral lymphoid tissues. B-cell development requires a variety of soluble factors, including IL-3, IL-4, IL-11, INFγ, and TGFβ (Burkhard, 2010). T-cell precursors migrate to the thymus during embryonic development, where they undergo proliferation, differentiation, and both positive and negative selection. T-cell development requires IL-7 stimulation (Burkhard, 2010). NK-cell development, which requires IL-15 stimulation, occurs mostly in the fetal liver and thymus, as well as in the bone marrow after birth (Burkhard, 2010).

In health, blood lymphocytes are predominantly T-cells. Similar to neutrophils, blood lymphocytes are divided into circulating and marginating pools, with cells frequently shifting between these pools. Lymphocytes in the blood travel to lymph nodes, where they exit the blood through high endothelial venules and enter the lymph node cortices. Lymphocytes that migrate through the lymph nodes leave through efferent lymphatic vessels, from which they return to the blood. Blood lymphocytes may also emigrate to other tissues if chemotactic stimuli are present. In tissues, lymphocytes may proliferate, die, or migrate back into blood. Lymphocyte life spans are highly variable depending on the cell type and function, and some lymphocytes may live for years (Stockham and Scott, 2008a).

Lymphocytes are the most numerous blood leukocytes in rats and mice, and are typically present in greater numbers than neutrophils in young cynomolgus monkeys and dogs. Cynomolgus monkeys used in nonclinical toxicology studies are often young or peripubertal, with greater lymphocyte than neutrophil counts. However, in both cynomolgus monkeys and dogs, there is a shift of blood leukocyte populations to a predominance of neutrophils with maturation, and nonclinical toxicology studies may include

animals having either hematologic pattern. In adults of species with neutrophils as the most numerous blood leukocyte, lymphocytes are typically the second most numerous.

12.11.2.2.1 Increases in lymphocyte counts (lymphocytosis)

12.11.2.2.1.1 Age-related

Lymphocytes are common as the predominant blood leukocyte of neonate and juvenile animals, even in species with predominant neutrophils in the circulation of adults. Recognition of this apparent "lymphocytosis" of young animals is important, as comparison of lymphocyte counts in young animals with adult historical control data may give the appearance of increased lymphocyte counts. Species in which "lymphocytosis" in young animals has been described include dogs and cats (Stockham and Scott, 2008a) and cynomolgus monkeys (Sugimoto et al., 1986). As the shift from predominantly lymphocytes to predominantly neutrophils in circulation occurs around 4 to 5 years old in cynomolgus monkeys (Sugimoto et al., 1986), it is not uncommon for nonclinical toxicology studies to include individuals with both lymphocyte and neutrophil-predominant leukograms.

12.11.2.2.1.2 Catecholamine-induced

Similar to increases in neutrophil counts, increases in endogenous or exogenous catecholamines associated with excitement, fear, or exercise result in transient increases in lymphocyte counts. Catecholamine-induced increases in lymphocyte counts are associated with rapid shifts from the marginating to circulating lymphocyte pool, which is thought to be due to both decreased lymphocyte adhesion to endothelial cells and increased blood flow (Benschop et al., 1996). Release of lymphocytes from the spleen in response to catecholamine stimulation likely also contributes to the increase in blood lymphocyte counts, but lymph nodes and bone marrow do not appear to be significant contributors (Benschop et al., 1996). There are species differences in the response of lymphocytes to catecholamines, and the resultant increases in lymphocyte tend to be more common in monkeys and cats, while less common in adult dogs (Smith et al., 2002; Schultze, 2010).

12.11.2.2.1.3 Decreased Glucocorticoids

Glucocorticoids have a negative effect on blood lymphocyte counts due to their effects on lymphocyte distribution in the body and suppression of lymphopoieis. Hypoadrenocorticism (Addison's disease), in which the adrenal glands are unable to maintain normal concentrations of glucocorticoids, may be associated with increases in blood lymphocyte counts due to the loss of the inhibitory effects of endogenous glucocorticoids (Oelkers, 1996; Stockham and Scott, 2008a; Avery and Avery, 2007).

12.11.2.2.1.4 Inflammation

Acute inflammatory processes tend to cause decreases in lymphocyte counts, but some acute infectious processes, particularly several viral infections, may cause increases in lymphocyte counts. However, increases in lymphocyte counts are commonly observed with chronic inflammatory processes. Chronic stimulation of lymphocytes with antigens or cytokines results in the increased production of lymphocytes with release into the blood, causing the increases in blood lymphocyte counts. Reactive lymphocytes may be observed in blood accompanying inflammation-induced increases in lymphocytes counts. Reactive lymphocytes have a spectrum of morphologic changes that include increased cytoplasmic basophilia, large cells with mildly increased amounts of cytoplasm (lower nuclear to cytoplasmic ratios), variable patterns of chromatin clumping, and variable numbers of visible nucleoli. Occasionally, reactive lymphocytes may have paranuclear cytoplasmic clearing, giving a plasmacytoid appearance. Infectious mononucleosis syndromes in people are a relatively common cause of an inflammatory or reactive increase in lymphocyte counts that are usually acute (Vasu and Caligiuri, 2016). Chronic infections leading to increase in blood lymphocyte counts may include visceral leishmaniasis, parasitic infections such as strongyloidiasis, and leprosy (Vasu and Caligiuri, 2016; Rai et al., 2008; Myers et al., 2000). Several chronic infections, including ehrlichiosis, Rocky Mountain spotted fever, leishmaniasis, trypanosomiasis, and brucellosis, have been associated with increases in blood lymphocyte counts in dogs (Schultze, 2010).

12.11.2.2.1.5 Neoplasia

Increases in blood lymphocyte counts associated with lymphoproliferative neoplasia may represent either lymphocytic leukemia or the leukemic phase of lymphoma. A normal circulating lymphocyte population should be heterogeneous, with predominantly small lymphocytes and fewer intermediate to large lymphocytes. Increased blood lymphocyte counts with a loss of heterogeneity in the blood lymphocyte population or predominantly a monomorphic intermediate to large lymphocyte population are key features for diagnosing lymphoproliferative neoplasia. With the exception of chronic lymphocytic leukemia, which is characterized by increased numbers of small lymphocytes with few or subtle morphologic alterations, circulating lymphocytes often have abnormal morphologic features that may be observed microscopically. Abnormal morphologic features of the leukemic lymphocyte population may include increased cytoplasmic basophilia, increased amounts of cytoplasm with altered nuclear to cytoplasmic ratios, irregular clumping of nuclear chromatin, indentation or lobulation of nuclei, variably sized but typically prominent nucleoli, or multiple nucleoli. Some of these morphologic features are similar to those observed in reactive lymphocytes, but these two processes may be distinguished by the overall heterogeneity of the lymphocyte population and proportion of the lymphocyte population with these morphologic alterations. Additional testing, such as flow cytometry for phenotyping or PCR for antigen receptor rearrangement (PARR), also aids in the diagnosis or characterization of lymphoproliferative neoplasia.

Lymphoproliferative neoplasia is observed as a relatively common background finding in older rats and mice during nonclinical toxicology studies (Frith et al., 1993). Although less common, it may also be observed in low frequencies in older monkeys. Monkeys with concurrent infection with species-specific lymphocryptoviruses and immunosuppression have been reported to have virus-related lymphoproliferative neoplasias (Magden et al., 2015). Lymphocryptoviruses are in the Gammaherpesvirinae subfamily and are related to Epstein-Barr virus, which has been associated with lymphoproliferative neoplasia in people but may aberrantly infect New World monkeys (Magden et al., 2015; Thorley-Lawson and Gross, 2004).

Increases in blood lymphocyte counts may also occur secondary to nonlymphoproliferative neoplasms. Increases in polyclonal T-cells have been reported in patients with malignant thymoma, while increases in reactive lymphocytes have been reported with AML and systemic mastocytosis (Vasu and Caligiuri, 2016).

12.11.2.2.1.6 Xenobiotic-induced

Xenobiotic-induced increases in blood lymphocyte counts are relatively uncommon, with most reports associated with an administration of catecholamines or rare idiosyncratic hypersensitivity-type reactions.

Administration of exogenous catecholamines, such as epinephrine, or adrenergic agonists has similar effects on blood lymphocyte counts as those mediated by endogenous catecholamines. Rapid shifting of lymphocytes from marginating to circulating blood pools as well as mobilization of lymphocytes from the spleen contributes to the increases in lymphocyte counts. Lymphocytes appear to have their adrenergic effects primarily mediated by β_2-adrenergic receptors (Benschop et al., 1996).

Increases in blood lymphocyte counts with the presence of atypical lymphocytes in circulation have been associated with drug reaction with eosinophilia and systemic symptoms (DRESS), a form of drug-related hypersensitivity. Several anticonvulsant drugs, including phenobarbital and phenytoin, allopurinol, minocycline, sulfonamides, gold salts, and dapsone have been associated with DRESS (Roujeau, 2005; Callot et al., 1996).

Treatment of CML and chronic lymphocytic leukemia with dasatinib and ibrutinib, respectively, has been associated with increases in blood lymphocytes counts (Vasu and Caligiuri, 2016). Dasatinib-related increases in lymphocyte counts may be related to expansion of T-cell or NK-cell populations and increases in IL-2R, INF-γ, and IL-6, with reported favorable outcome to treatment, while ibrutinib-related increases in lymphocyte counts may be related to and increased efflux of lymphocytes from lymphoid tissues (Vasu and Caligiuri, 2016).

12.11.2.2.2 Decreases in lymphocyte counts (lymphopenia)

12.11.2.2.2.1 Glucocorticoid-induced

Endogenous glucocorticoids may be increased with chronic stress or hyperadrenocorticism, and decreases in lymphocyte counts tend to be the most prominent and consistent glucocorticoid-mediated leukocyte change across species. Glucocorticoids induce decreases in blood lymphocyte counts through several mechanisms. In addition to a rapid shift of lymphocytes from the circulating to marginating and tissue pools, there is evidence for both lymphocyte redistribution from blood to bone marrow (Fauci, 1975) and decreased efflux of lymphocytes from lymphoid tissues (Bloemena et al., 1990) contributing to the shift of lymphocytes to tissue pools. With long-term increases in glucocorticoid concentrations, lymphotoxicity may be observed due to an increased activation of apoptotic pathways (Garvy et al., 1993; Tuckermann et al., 2005). In rats, feed restriction has been associated with decreases in blood lymphocyte counts and lymphocyte depletion in various lymphoid tissues (Moriyama et al., 2008), possibly associated with prolonged stress and therefore a glucocorticoid-mediated effect. Indirect test article-related effects mediated by altered food consumption are important to consider in nonclinical toxicology studies, in which test articles may cause direct or indirect hematologic effects. Interpretation of these changes, including stress-associated effects, must be made cautiously and thoughtfully, using a weight of evidence approach.

12.11.2.2.2.2 Inflammation

Decreases in lymphocyte counts are typically observed with acute inflammation. These decreases are likely due to increased margination and emigration of lymphocytes to the site of inflammation, increased migration of lymphocytes to lymphoid tissues, and decreased efflux of lymphocytes out of lymphoid tissues (Stockham and Scott, 2008a). Stress associated with illness or acute inflammation may also contribute by glucocorticoid-induced mechanisms (Stockham and Scott, 2008a; Schultze, 2010).

Many infectious agents may cause a decrease in lymphocyte counts due to inflammation. Infectious agents associated with decreases in lymphocyte counts include viruses such as coronavirus, parvovirus, West Nile virus, hepatitis viruses, and influenza (Vasu and Caligiuri, 2016; Schultze, 2010); acute systemic bacterial infections; as well as tuberculosis, typhoid fever, and bacterial pneumonias (Vasu and Caligiuri, 2016; Magden et al., 2015). The acute phase of malaria may also be associated with decreases in lymphocyte counts (Vasu and Caligiuri, 2016).

12.11.2.2.2.3 Viral-induced

Infection with immunodeficiency viruses, including human, simian, and feline immunodeficiency viruses, may result in destruction of both infected and noninfected lymphocytes. HIV directly infects $CD4^+$ T-cells via the CD4 molecule; infected cells then migrate to lymphoid tissues where the virus replicates and infects more $CD4^+$ T-cells (Chinen and Shearer, 2010). HIV-mediated lymphocyte destruction is likely multifactorial; HIV may be cytotoxic, directly induce T-cell apoptosis, induce T-cell death through a nonspecific immune response, and cause T-cell death by stimulating autophagocytic pathways (Chinen and Shearer, 2010; Stump and VandeWoude, 2007). SIV and FIV tropism for T-cells is also mediated by receptors expressed by $CD4^+$ T-cells (Stump and

VandeWoude, 2007). Simian betaretrovirus also causes decreases in blood lymphocyte counts due to infection and eventual depletion of both B-cells and T-cells, although infection of nonlymphoid cells also occurs (Montiel, 2010).

12.11.2.2.2.4 Immune-mediated

Immune-mediated destruction of lymphocytes is uncommon. When occurring in the autoimmune disease SLE, such immune-mediated decreases in lymphocyte counts typically occur with concurrent cutaneous, arthritic, or neurologic disorders (Stone, 2005), and may be the result of autoantibodies causing lymphocyte destruction or death through apoptosis (Lu et al., 2012; Massardo et al., 2009; Noguchi et al., 1992; Budman and Steinberg, 1977).

12.11.2.2.2.5 Inherited causes

Although rare, some inherited immunodeficiency syndromes cause blood lymphocyte counts to be decreased. One example, severe combined immunodeficiency (SCID), has been reported in humans, dogs, mice, and horses (Suter, 2010; Notarangelo, 2010; Meek et al., 2001; Felsberg et al., 1992; Custer et al., 1985). SCID may be inherited through autosomal recessive or X-linked recessive patterns, and causes consistent decreases in T-cells, with concurrent decreases in B-cells or NK-cells in some forms of the disease. SCID in humans is caused by a variety of mechanisms, including: adenosine deaminase deficiency resulting in early cell death due to metabolite accumulation; common gamma chain or janus kinase 3 (JAK3) mutations that cause decreased survival of T-cell precursors due to defective cytokine signaling; recombinase-activating gene 1 (RAG1) or RAG2 mutations that cause defective V(D)J rearrangement of B-cell and T-cell receptors; and mutations in CD3 or CD45 that cause defects in T-cell receptor signaling (Suter, 2010; Notarangelo, 2010). SCID in Jack Russell terriers, Arabian foals, and mice has been demonstrated to be caused by defective V(D)J recombination due to loss of DNA-dependent protein kinase (Meek et al., 2001). X-linked SCID has been described in both Bassett Hounds and Welsh Corgi dogs (Suter, 2010). Other inherited immunodeficiency syndromes resulting in decreases in blood lymphocyte counts include reticular dysgenesis, ataxia-telangiectasia, and Wiskott-Aldrich syndrome (Vasu and Caligiuri, 2016).

12.11.2.2.2.6 Loss of lymph fluid

Although uncommon, disorders causing chronic loss of lymphocyte-rich lymph fluid lead to body depletion of lymphocytes and decrease in blood lymphocyte counts. Examples of such conditions include protein-losing enteropathy, lymphangiectasia, ulcerative enteritis, or repeated iatrogenic removal of chylothoracic fluid (Vasu and Caligiuri, 2016; Schultze, 2010; Stockham and Scott, 2008a).

12.11.2.2.2.7 Neoplasia

Although lymphoproliferative neoplasia may be associated with increases in lymphocyte counts as previously discussed, lymphoma and lymphocytic leukemia may also be associated with decreases in lymphocyte counts including from altered lymphocyte recirculation patterns or decreased production of nonneoplasic lymphocytes secondary to lymphoid organ damage (Mitrovic et al., 2012; Schultze, 2010; Stockham and Scott, 2008a).

12.11.2.2.2.8 Xenobiotic-induced

Lymphoid suppression is a common finding with many xenobiotics and may be associated with decreases in blood lymphocyte counts. Mechanisms through which these decreases occur may either be part of the expected pharmacology of these compounds or may represent an off-target effect. Several examples of xenobiotic-induced decreases in lymphocyte counts are described here.

Administration of exogenous glucocorticoids, for antiinflammatory or immunosuppressive purposes, will result in decreases in blood lymphocyte counts. The mechanisms for this are the same as those for endogenous glucocorticoids, and include altered blood and tissue pool distribution, decreased efflux of lymphocytes from lymphoid tissues, and increased lymphocyte apoptosis with prolonged glucocorticoid exposure.

Other immunosuppressive agents that cause decreased blood lymphocyte counts include cyclophosphamide, methotrexate, purine nucleoside analogs, and azathioprine. Cyclophosphamide has been associated with profound decrease in blood lymphocyte counts through its effects on all lymphocyte subtypes (Gergely, 1999). Methotrexate causes decreases in circulating $CD4^+$ and $CD8^+$ T-cells, while cladribine, a purine nucleoside analog, causes apoptosis of lymphocytes and has been reported to affect both $CD4^+$ and $CD8^+$ T-cells (Gergely, 1999). Azothiaprine-induced decreases in blood lymphocyte counts appear to be due to long-term administration at high dose levels (Johnson et al., 1995; Gergely, 1999).

As discussed previously, numerous xenobiotics have been associated with drug-induced SLE in people. Decreases in lymphocytes in these cases are likely due to the production of autoantibodies with subsequent lymphocyte destruction, similar to nonxenobiotic-induced SLE. Xenobiotics associated with SLE include several anticonvulsants such as phenothiazines, chlorpromazine, and valproate, several antibiotics such as penicillin, streptomycin, tetracycline, griseofulvin, and sulphonamides, and miscellaneous xenobiotics such as captopril, phenylbutazone, and lovastatin (Stone, 2005; Mutasim and Adams, 2000).

Chemotherapeutic agents are also frequently associated with decreases in lymphocyte counts, which may precede episodes of febrile neutropenia (Gergely, 1999). Carboplatin, dacarbazine, and paclitaxel have been reported to induce decreases in $CD4^+$ T-cells, while epirubicin and mitomycin appear to affect $CD8^+$ T-cells to a greater degree than $CD4^+$ T-cells, and pentostatin affects both B-cell and T-cell populations (Gergely, 1999).

Antilymphocyte monoclonal antibodies have been used to treat autoimmune diseases as well as to cause immunosuppression to prevent acute transplant rejection. Examples of these monoclonal antibodies include Muromonab CD3 (OKT3) and CAMPATH-1H (Vial et al., 2002; Gergely, 1999).

Other classes of drugs reported to cause decrease lymphocyte counts are varied: pesticides including organochloride pesticides such as pentachlorophenol, organotin compounds, and organophosphates (Corsini et al., 2013); thienopyridines, such as clopidogrel and ticlopidine, which can cause direct lymphotoxicity at high concentrations (Maseneni et al., 2013); the histamine H2 receptor antagonist cimetidine; the anticonvulsant carbamazepine; imidazoles used to treat fungal infections; and opioids such as morphine (Gergely, 1999).

12.11.2.3 Monocytes

Bone marrow common myeloid progenitors differentiate into the monocytic lineage under stimulation by stem cell factor, GM-CSF, macrophage-colony-stimulating factor (M-CSF), IL-6, IL-1, and IL-3 (Papenfuss, 2010). Monoblasts further differentiate to promonocytes and then to monocytes.

Blood monocytes are distributed in both circulating and marginating pools, and marginating pool monocytes can emigrate into tissues. Circulating half-life of monocytes in mice has been reported to range from 24 to 60 h (Provencher Bolliger et al., 2010). Macrophages and dendritic cells can arise from monocytes or monocyte precursors depending on the local tissue microenvironment and cytokine stimulation (Papenfuss, 2010), forming the mononuclear phagocytic system.

Although present in low numbers, monocytes represent the third most numerous blood leukocytes in health, after neutrophils and lymphocytes. They are typically the largest of the leukocytes in routine blood films. Monocytes play a major role in resolution of infectious processes, particularly those involving larger or more complex organisms, such as fungi and protozoa, and in the clearance of other foreign material from the body. Following cardiac blood collection in mice, tissue macrophages may be inadvertently collected and may rarely be observed during blood smear evaluation.

12.11.2.3.1 Increases in monocyte counts (monocytosis)

12.11.2.3.1.1 Catecholamine-induced

Although less commonly observed than increases in neutrophil or lymphocyte counts, blood monocyte counts may be modestly increased under the stimulation of endogenous catecholamines. This effect is most likely mediated by rapid shifting of monocytes from the marginating pool to the circulating pool (Everds et al., 2013a).

12.11.2.3.1.2 Glucocorticoid-induced

Endogenous glucocorticoids classically cause increases in blood monocyte counts. However, these increases in monocyte counts are less consistently observed than decreases in lymphocyte counts or increases in neutrophil counts, and no perceptible change in monocyte counts may occur (Hall, 2013; Everds et al., 2013a). There have also been reports of decreases in monocyte counts attributable to endogenous glucocorticoids, which may be associated with decreased production with prolonged glucocorticoid exposure (Thompson and van Furth, 1973), or may be transient and followed by an increased in monocyte counts (Steer et al., 1997; Rinehart et al., 1975).

12.11.2.3.1.3 Inflammation

Increases in blood monocyte counts occur with both acute and chronic inflammation, and such inflammatory increases in monocytes may be associated with both infectious and noninfectious etiologies. Tuberculosis and other mycobacterial infections are commonly associated with increases in monocyte counts which may represent an increased tissue demand for macrophages (Lichtman, 2016a). Increases in monocyte counts have also been associated with bacterial endocarditis and sepsis, osteomyelitis, various pyogranulomatous diseases, candidiasis, viral infections including cytomegalovirus and influenza, and several parasitic diseases including pneumocystosis, Entopolypoides macacai infection in old world monkeys and apes, and dirofilariasis in dogs (Lichtman, 2016a; Magden et al., 2015; Schultze, 2010). However, increases in monocyte counts may be less commonly related to malaria, leishmaniasis, and rickettsial diseases in people than previously thought (Lichtman, 2016a). Noninfectious causes of increases in blood monocyte counts include inflammatory bowel disease, ulcerative gastritis, myocardial infarction, and parturition (Lichtman, 2016a).

12.11.2.3.1.4 Immune disorders

Numerous immune disorders are associated with increases in blood monocyte counts. Immune-mediated destruction of erythrocytes or neutrophils often has concurrent increases in monocyte counts (Schultze, 2010; Stockham and Scott, 2008a). The increase in monocyte count associated with immune-mediated neutropenia may be due to cytokine stimulation of the common precursor of both granulocytes and monocytes (Stockham and Scott, 2008a). Rebound increases in neutrophil counts during recovery from agranulocytosis often have concurrent increases in monocyte counts (Lichtman, 2016a; Schultze, 2010), which may also be due to stimulation of the common granulocyte and monocyte precursor. Systemic lupus erythematosus (Budman and Steinberg, 1977), rheumatoid arthritis (Buchan et al., 1985), sarcoidosis (Goodwin et al., 1979), and other connective tissue diseases (Lichtman, 2016a) may also cause increases in monocyte counts.

12.11.2.3.1.5 Neoplasia

Hematopoietic malignancies involving the monocytic lineage are rare, but are commonly associated with increased blood monocyte counts. These hematopoietic neoplasms include myelodysplastic syndromes (e.g., chronic myelomonocytic leukemia), acute monocytic leukemia, acute myelomonocytic leukemia, dendritic cell leukemia, and malignant histiocytosis (Schultze, 2010; Tefferi and Vardiman, 2009; Villeneuve et al., 2008; Sun et al., 2007; Lichtman and Segel, 2005; Castoldi and Rigolin, 2001; Rigolin et al., 1997; Laurencet et al., 1994). Blood monocytes associated with these hematopoietic neoplasms frequently have abnormal morphologic features and may be accompanied by monoblasts or promonocytes in circulation.

Increases in monocyte counts related to neoplasia are not limited to hematopoietic neoplasms of the monocytic lineage. Increases in monocyte counts have also been described in association with a wide spectrum of lymphoproliferative neoplasms, soft tissue sarcomas, hemangiosarcomas, chondrosarcomas, rectal polyps, and colon cancer (Lichtman, 2016a; Schultze, 2010; Melichar et al., 2001; Ruka et al., 2001). In one study, over half of the patients with solid tumor malignancies were reported to have concurrent increases in monocyte counts, which were independent of tumor metastasis (Barrett, 1970).

12.11.2.3.1.6 Xenobiotic-induced

Administration of exogenous glucocorticoids classically causes increases in monocyte counts, although this effect may be inconsistently observed and no change in blood monocyte counts may occur. Some studies have demonstrated that transient decreases in monocyte counts occur immediately following administration of exogenous glucocorticoids with subsequent increases in monocyte counts (Rinehart et al., 1975; Steer et al., 1997), but others have only reported increases in monocyte counts with exogenous glucocorticoid administration (Barker et al., 2012).

Administration of exogenous cytokines may also result in increases in blood monocyte counts. Such increases in blood monocyte counts have been observed with G-CSF (Ranaghan et al., 1998; Liu et al., 2004), GM-CSF (Schmitz et al., 1994), M-CSF (Weiner et al., 1994; Cole et al., 1994), or IL-10 administration (Chernoff et al., 1995). M-CSF administration-related increases in monocyte counts have been associated with concurrent, dose-limiting decreases in platelet counts (Weiner et al., 1994; Cole et al., 1994).

Increases in monocyte counts have also been reported in a few patients associated with pseudolymphoma syndrome caused by therapy with several anticonvulsants, including phenytoin, phenobarbital, and valproic acid (Choi et al., 2003).

12.11.2.3.2 Decreases in monocyte counts (monocytopenia)

Decreases in monocyte counts may be difficult to detect due to the relatively low blood monocyte counts in humans and most laboratory animal species, particularly if reference intervals or historical control ranges, rather than values from a concurrent control group, are being used for determining if changes in blood monocyte counts are present. In nonclinical toxicology studies, comparison to concurrent controls or pretest may enable detection of more subtle decreases in monocyte counts.

12.11.2.3.2.1 Immune-mediated

Immune-mediated decreases in monocyte counts are typically not observed in isolation and are associated with causes of pancytopenia, such as aplastic anemia. With aplastic anemia, destruction of early hematopoietic precursors results in loss of production of most or all hematopoietic cell lineages and subsequent development of severe decreases in multiple blood component counts. Aplastic anemia can also occur with nonimmune-mediated conditions, such as anorexia nervosa (Abella et al., 2002).

12.11.2.3.2.2 Inherited causes

Uncommonly there are cases of inherited marked decreases in blood monocyte counts. In humans, an autosomal dominant inheritance pattern has been associated with decreased monocyte counts with a resulting increased susceptibility to mycobacteria and a variety of other infectious agents (Vinh et al., 2010). Mutations in GATA2, a transcription factor that regulates hematopoietic cell gene expression and integrity, have been reported as a cause for autosomal dominant decreases in blood monocyte counts (Camargo et al., 2013; Hsu et al., 2011).

12.11.2.3.2.3 Neoplastic

Decreases in blood monocyte counts may occur secondary to hematologic malignancies or metastatic nonhematology malignancies that efface the bone marrow. Neoplastic myelophthisis results in decreased production of normal hematopoietic cells and therefore blood component counts, including monocytes. Examples of such reported conditions include hairy cell leukemia (den Ottolander et al., 1983; Ratain et al, 1985) and chronic lymphocytic leukemia (De Rossi et al., 1991).

12.11.2.3.2.4 Xenobiotic-induced

Xenobiotic-induced bone marrow suppression can often cause decreases in blood monocyte counts in combination with decreases in other blood component counts. Causes include chemotherapeutic agents, such as discussed in xenobiotic-induced decreases in neutrophil counts, due to direct hematopoietic precursor cell toxicity and xenobiotics associated with aplastic anemia, such as chloramphenicol. Monocyte cytotoxicity has been reported with methylmetacrylate monomer used in joint replacement surgery (Dahl et al., 1994). Lindane, a pesticide, has been reported to cause CFU-GM cytotoxicity (Parent-Massin and Thouvenot, 1993).

12.11.2.4 Eosinophils

Eosinophils share a common early myeloid precursor with neutrophils. Early proliferation of the eosinophil lineage appears to be largely due to stimulation of myeloid precursors with GM-CSF and IL-3, while IL-5 plays a critical role in terminal eosinophil differentiation and maturation (Sanderson, 1992).

Similar to other granulocytes, eosinophils are distributed into proliferating and maturing pools in the bone marrow, and into circulating and marginating pools in the blood. The bone marrow is the primary site of eosinophil production, although rat eosinophils migrate and complete their maturation in the spleen (Young and Meadows, 2010). Early proliferating bone marrow eosinophils are usually indistinguishable microscopically from other early myeloid precursors because their characteristic granules do not begin to form until the late promyelocyte stage (Radin and Wellman, 2010). The time for production of mature eosinophils from the myeloblast stage is 2–6 days and the half-life of mature eosinophils in circulation ranges from less than 1 to 24 h, although both of these transit times vary by species (Young and Meadows, 2010). Eosinophils migrate into tissue from circulation, where they live for about 2 days unless stimulation occurs (Young and Meadows, 2010).

Eosinophil counts are normally low in most species, which can make detection of decreases in eosinophil counts difficult. Similar to detecting decreases in monocyte counts, comparison of treated groups with concurrent controls or pretest values in nonclinical toxicology studies will aid in the identification of changes in eosinophil counts. Eosinophils are morphologically distinct from other leukocytes due to their large, pink-staining cytoplasmic granules; however, species differences in granule size, shape, and staining properties exist. Blood eosinophil counts in health generally only exceed basophil counts.

12.11.2.4.1 Increases in eosinophil counts (eosinophilia)

12.11.2.4.1.1 Decreased glucocorticoids

Although uncommon, decreases in endogenous glucocorticoid concentrations due to hypoadrenocorticism have been associated with mild increases in blood eosinophil counts (Wardlaw, 2016; Stockham and Scott, 2008a). This effect is most likely due to the loss of glucocorticoid-associated shifting of blood eosinophils to the marginating pool as well as the lack of proapoptotic stimulation of glucocorticoids on eosinophil precursors.

12.11.2.4.1.2 Inflammation and Hypersensitivity

Both acute and chronic inflammatory stimulation may result in increases in blood eosinophil counts along with increases in neutrophil, lymphocyte, and/or monocyte counts. However, inflammatory processes that stimulate primarily an eosinophil response may also occur. IL-5, eotaxin, and RANTES are cytokines and chemokines that selectively stimulate eosinophil responses (Sampson, 2000). Some of the most common inflammatory processes involving eosinophils include parasite and allergy-induced inflammation.

Parasitism is considered the most common cause of increases in blood eosinophil counts worldwide (Wardlaw, 2016), of which helminth (e.g., nematode, trematode, or cestode) infections are the major cause (Tefferi et al., 2006; Leder and Weller, 2000). Inflammatory increases in eosinophil counts in response to helminths are cytokine (e.g., IL-5) mediated (Valent, 2009), but IgE and histamine release from mast cells, anaphylatoxin (e.g., C5a) production from complement activation, helper T-cell activation, and direct stimulation of eosinophils with helminthic antigens also play a role in eosinophil recruitment (Wardlaw, 2016; Leder and Weller, 2000; McEwen, 1992). Helminth migration through host tissues is a key factor in stimulating increases in blood eosinophils and tissue eosinophilic inflammation, and helminths that remain confined to the intestinal lumen may not result in an eosinophil response (Leder and Weller, 2000). The degree of the eosinophil response and increases in blood eosinophil counts are also dependent on parasite burden, maturation, and distribution in tissues (Leder and Weller, 2000). Ascariasis, strongyloidiasis, trichinosis, filariasis, and ancylosomiasis have all been associated with increases in eosinophil counts in humans (Wardlaw, 2016; Tefferi et al., 2006; Leder and Weller, 2000), and many of these may also be observed in common laboratory species (Schultze, 2010; Magden et al., 2015; Korenaga et al., 1991; Ogilvie et al., 1980). Helminth infection in most purpose-bred laboratory animals is uncommonly observed during nonclinical toxicology studies due to breeding and housing facility biosecurity measures, but several less commonly used large animal species from other sources, such as farm pigs, calves, and sheep, have helminth infections more frequently observed.

Infection with nonhelminth parasites may also cause increases in blood eosinophil counts. Ectoparasites, including fleas and ticks, have been associated with increase in eosinophil counts in dogs and cats and may be due to arthropod-related allergic or hypersensitivity reactions (Schultze, 2010; Valenciano et al., 2010; Stockham and Scott, 2008a). Several protozoal infections, including isosporiasis (Jongwutiwes et al., 2002; Junod, 1987) and toxoplasmosis (Grant and Klein, 1987), may result in increases in blood eosinophil counts. However, protozoal agents that infect erythrocytes, such as *Plasmodium* and *Babesia* species, are generally not expected to result in altered blood eosinophil counts (Tefferi et al., 2006; Stockham and Scott, 2008a). Some bacterial infections, including borreliosis (Granter et al., 1996) and rickettsiosis (Wardlaw, 2016), and several viral infections, including herpes virus and HIV (Wardlaw, 2016; Tietz et al., 1997), have also been associated with increases in blood eosinophil counts. Fungal infections that cause allergic inflammation, such as coccidiomycosis, candidiasis, and aspergillosis (Wardlaw, 2016), may also cause increases in blood eosinophil counts.

Allergic inflammation is another common cause of increases in blood eosinophil counts. Allergic conditions associated with increases in blood eosinophil counts include asthma, atopic dermatitis, and allergic rhinitis although increases in eosinophil counts with these conditions are usually mild (Wardlaw, 2016). Allergic inflammation associated with immediate release of IgE is

considered a type I hypersensitivity reaction. Some allergen-induced inflammation may be attributable to type IV (delayed-type or cell-mediated) hypersensitivity following T_H activation with subsequent eosinophil recruitment. However, some differences have been observed between atopic dermatitis and classic type IV hypersensitivity (Gaga et al., 1991), so not all T_H-mediated allergic inflammation may represent a true type IV hypersensitivity reaction. As with most forms of inflammatory increases in blood eosinophil counts, production of cytokines and chemokines, such as IL-5 and eotaxin, appear to play a major role. In allergic asthma, activated T-helper type 2 (T_H2) cells release or stimulate the release of these cytokines and chemokines, resulting in recruitment and activation of eosinophils (Rosenberg et al., 2013). Sensitization of the airways to ovalbumin with subsequent challenge in mice has mimicked many of the clinical and pathological features of allergic asthma, including the interaction of T_H2 cells and eosinophils (Rosenberg et al., 2013). However, asthma encompasses a heterogeneous set of phenotypes and not all forms demonstrate clinical improvement in response to therapies targeting IL-5 or eosinophils (Wegmann, 2011). While asthma is uncommon in most laboratory animal species, it can be experimentally induced in mice and may occur naturally in cats. Activation of T_H2 and T_H1 cells have been proposed to contribute to the pathology of atopic dermatitis, with the T_H2 activation being of particular relevance to the development of increases in blood eosinophil counts (Grewe et al., 1998), similar to allergic asthma. Activation of T_H2 cells also plays a role in cytokine and chemokine elaboration with eosinophil recruitment in allergic rhinitis, although effects on eosinophils in allergic rhinitis are also mediated by histamine and IgE release from mast cells and histamine release from basophils (Borish, 2003).

Inflammatory increases in blood eosinophil counts can also be associated with paraneoplastic syndromes, likely related to increases in IL-5, which may be liberated by activated T_H cells or directly by the neoplasm. Lymphoma, including both T- and B-cell lymphomas, is a common cause of paraneoplastic increases in eosinophil counts in multiple species, including humans, dogs, cats, and horses (Wardlaw, 2016; Davis and Rothenberg, 2014; Valent, 2009; Schultze, 2010; Valenciano et al., 2010; Stockham and Scott, 2008a; Marchetti et al., 2005; Cave et al., 2004; Duckett and Matthews, 1997). However, many nonlymphoid tumors have also been associated with paraneoplastic increases in blood eosinophil counts, including mammary carcinoma, hepatocellular carcinoma, squamous cell carcinoma, thymoma, nonsmall-cell lung cancer, and mast cell diseases including systemic mastocytosis and mast cell leukemia (Schultze, 2010; Balian et al., 2001; Walter et al., 2002; Pandit et al., 2007; Valent, 2009).

12.11.2.4.1.3 Neoplastic

Myeloid neoplasms can result in clonal eosinophil expansion and increases in blood eosinophil counts expansion (Tefferi et al., 2006). Neoplastic increases in eosinophil counts have been associated with acute eosinophilic leukemia, chronic eosinophilic leukemia, chronic myeloid leukemia, and myelodysplastic syndrome (Wardlaw, 2016; Tefferi et al., 2006; Schultze, 2010). Clonal increases in eosinophil counts may be difficult to distinguish from idiopathic hypereosinophilic syndrome, and cytogenetic analysis may be necessary; numerous cytogenetic abnormalities have been reported with clonal increases in eosinophil counts (Tefferi et al., 2006).

12.11.2.4.1.4 Idiopathic

There are numerous reports of idiopathic increases in blood eosinophil counts. Such conditions include eosinophilic esophagitis, eosinophilic gastroenteritis, eosinophilic myositis, eosinophilic cellulitis, and eosinophilic pneumonitis in people, dogs, and/or cats (Wardlaw, 2016; Stockham and Scott, 2008a). Hypereosinophilic syndrome (HES) in people is another condition that falls under the umbrella of idiopathic increases in eosinophil counts. In HES, chronic increases in eosinophil counts are observed without evidence of an underlying causative condition. This condition is associated with marked tissue infiltration and eventual organ damage and failure (Wardlaw, 2016; Rosenberg et al., 2013). However, some patients with HES eventually develop either a lymphoid or myeloid neoplasm (Wardlaw, 2016).

12.11.2.4.1.5 Spurious

In mice, automated hematology analyzer-generated blood eosinophil counts may be falsely elevated by large platelet clumps present in the specimen (O'Connell et al., 2015). Platelet clumping in mice is extremely common, and blood smear evaluation is often necessary to confirm the automated leukocyte differential count.

12.11.2.4.1.6 Xenobiotic-induced

Beta adrenergic blocking agents may be associated with modest increases in eosinophil counts, and administration of propranolol has been demonstrated to prevent catecholamine-induced decreases in eosinophil counts (Reed et al., 1970; Koch-Weser, 1968). The antibiotic tetracycline has been associated with increased eosinophil counts in dogs (Domina et al., 1997) and humans (Ho et al., 1979). Therapeutic administration of IL-2 for renal cell carcinoma has also been reported to cause increased blood eosinophil counts (Wardlaw, 2016). Administration of G-CSF and GM-CSF will also cause increases in blood eosinophil counts due to stimulation of common myeloid precursor proliferation. However, increases in eosinophil counts with these compounds will be small in comparison with the increases in blood neutrophil counts.

Numerous reactions to xenobiotics can also cause increases in blood eosinophil counts. Acute generalized exanthematous pustulosis due to drugs such as aminopenicillins and diltiazem, as discussed with xenobiotic-induced increases in neutrophil counts, may be associated with concurrent increases in eosinophil counts (Roujeau, 2005). Drug reaction with eosinophilia and systemic syndromes (DRESS) is a predominantly cutaneous manifestation of a drug hypersensitivity reaction. Numerous

compounds have been associated with DRESS, including several anticonvulsant drugs, such as phenobarbital and phenytoin, allopurinol, minocycline, sulfonamides, gold salts, dapsone, and spironolactone (Roujeau, 2005; Callot et al., 1996; Tefferi et al., 2006; Ghislain et al., 2004).

12.11.2.4.2 Decreases in eosinophil counts (eosinopenia)

12.11.2.4.2.1 Catecholamine-induced

In contrast to neutrophil, lymphocyte, and monocyte counts, blood eosinophil counts decrease in response to increased endogenous catecholamines. These effects may be inconsistent and difficult to detect due to timing of blood collection relative to the rapid changes in eosinophil counts and the normally low blood eosinophil counts. The β-adrenergic effects of epinephrine are believed to be the cause of the decreases in blood eosinophil counts (Koch-Weser, 1968). Catecholamines may also cause decreased release of eosinophils from bone marrow (McEwen, 1992).

12.11.2.4.2.2 Glucocorticoid-induced

Decreases in blood eosinophil counts are a classic feature of a glucocorticoid leukogram occurring in conjunction with decreases in lymphocyte counts and usually with increases in neutrophil counts. Blood eosinophils appear to be particularly responsive to the effects of glucocorticoids, and concurrent decreases in blood lymphocyte and eosinophil counts in common laboratory species used in nonclinical toxicology studies are a good indicator of stress (Hall, 2013). Glucocorticoids cause shifts in blood eosinophils from the circulating to the marginating pool as well as decreased release of eosinophils from the bone marrow (McEwen, 1992), and may also contribute to decreases in blood eosinophil counts from inhibition of prosurvival stimulation and direct induction of apoptosis (Druilhe et al., 2003; Wallen et al., 1991).

12.11.2.4.2.3 Inflammation

Severe acute or overwhelming inflammation, such as associated with sepsis, may cause eosinopenia in conjunction with neutropenia. Studies in mice have demonstrated that the decrease in blood eosinophils associated with severe acute inflammation occurs more rapidly than increases in glucocorticoids (Bass, 1975), and injection of material from an inflammatory exudate to adrenalectomized mice still resulted in deceases in eosinophil counts (Bass, 1977), indicating the mechanism of eosinophil decrease in acute inflammation is independent of adrenal function. It is believed that acute inflammatory decreases in blood eosinophil counts are due to shifting of eosinophils from the circulating to the marginating pool and subsequent egress into tissues in response to chemotactic stimuli. Acute inflammation associated with fungal and viral infections also tends to cause decreases in eosinophil counts (Leder and Weller, 2000).

12.11.2.4.2.4 Xenobiotic-induced

Although decreases in eosinophil counts are relatively uncommon with the exception of the administration of exogenous glucocorticoid-based xenobiotics, they may also be observed in cases of xenobiotic-induced bone marrow suppression and aplastic anemia. In these situations, the decreases in eosinophil counts do not occur in isolation but are generally observed with concurrent decreases in neutrophil, lymphocyte, and/or monocyte counts. Xenobiotic causes of bone marrow suppression classically include chemotherapeutic agents, while xenobiotics that can sporadically be associated with aplastic anemia include chloramphenicol and anticonvulsants such as phenytoin. Other xenobiotics associated with bone marrow suppression or aplastic anemia are described in more detail in the previous leukocyte subtype sections.

12.11.2.5 Basophils

Basophils develop in the bone marrow from uncommitted myeloid progenitor cells that differentiate into committed basophil progenitors. However, intermediate stages in basophil production have not been definitively identified, and there is evidence that basophils may share a common precursor with eosinophil, mast cells, or megakaryocytes (Radin and Wellman, 2010; Arock et al., 2002). Stimulation with IL-3 plays a major role in the terminal differentiation of basophils, while GM-CSF and IL-5 also play a role in basophil differentiation (Arock et al., 2002; Mayer et al., 1989). There is also some evidence for stem cell factor (SCF) and IL-4 stimulation in basophil differentiation (Pohlman, 2010; Favre et al., 1990). As studies in mice have shown that normal blood basophil counts may be maintained in the absence of IL-3, a required role for IL-3 in basophil production is not apparent (Lantz et al., 1998). Specific factors leading to terminal differentiation have not been identified for the basophil lineage, and basophil differentiation may in fact represent a default leukocyte differentiation pathway (Arock et al., 2002).

In blood, basophils are distributed into circulating and marginating pools, similar to other granulocytes. The circulating half-life of basophils is short (about 6 h), and they rapidly migrate into tissues where they have a much longer survival (up to 2 weeks) (Hirai et al., 1997; Pohlman, 2010).

Basophils are the least numerous leukocyte in blood, and in health usually compose approximately 0.5% or less of the blood leukocyte differential (Pohlman, 2010; Galli et al., 2016). Automated hematology analyzer differentials may provide low estimates of actual blood basophil counts in humans, and flow cytometric methods may provide a more accurate estimate (Meintker et al., 2013; Amundsen et al., 2012; Ducrest et al., 2005). Automated hematology analyzer counts in dogs and cats have been demonstrated to be inaccurate (Pohlman, 2010; Lilliehöök and Tvedten, 2011; Tvedten and Lilliehöök, 2011). However, basophils in rabbits appear to be detected with automated methods (Lilliehöök and Tvedten, 2011). Due to the evidence for low or inaccurate

basophil counts in humans, dogs, and cats, it is unclear how accurate automated basophil counts are in nonhuman primates and rodents used in nonclinical toxicology studies.

12.11.2.5.1 Increases in basophil counts (basophilia)

12.11.2.5.1.1 Inflammation

Increases in blood basophil counts may be associated with inflammatory stimuli, although decreases in basophil counts are more commonly observed (Galli et al., 2016). Increases in basophil counts have been associated with infectious, allergic, and paraneoplastic inflammatory conditions.

Parasitism is a relatively frequent cause of inflammatory increases in basophil counts, which are almost always observed in conjunction with increases in blood eosinophil counts. Many endoparasites, predominantly helminths with tissue exposure or migration, and ectoparasites, including a variety of arthropods, have been associated with concurrent increase in eosinophil and basophil counts (Schultze, 2010; Pohlman, 2010; Voehringer, 2009; Falcone et al., 2001; Brown and Rosalsky, 1984; Roth and Levy, 1980; Ogilvie et al., 1980).

Infectious agents other than parasites have also been reported to cause increases in basophil counts. Several viral etiologies associated with increases in basophil counts in humans include influenza, chickenpox, and smallpox viruses (Galli et al., 2016). Several bacterial infections may also cause increases in blood basophil counts, including tuberculosis (Galli et al., 2016) and infection with *Helicobacter pylori* (Karttunen et al., 1996).

Allergic inflammation that involves IgE and/or causes increases in eosinophil counts typically also causes increases in blood basophil counts. Immediate or delayed hypersensitivity may cause increases in basophil counts, although immediate hypersensitivity has also been associated with decreases in basophil counts in some cases; whether basophil counts increase or decrease may represent a distinction between allergic sensitization and an immediate allergic reaction (Shelley and Parnes, 1965). Food or inhalant allergies, such as ragweed pollen (Otsuka et al., 1986), can frequently cause increases in blood basophil counts (Galli et al., 2016; Pohlman, 2010). Allergic inflammation also occurs with insect stings or bites (Stockham and Scott, 2008a) and probably *H. pylori* infection (Karttunen et al., 1996).

Paraneoplastic increases in basophil counts have been associated with several neoplastic processes, including disseminated mast cell neoplasia (Schultze, 2010; Stockham and Scott, 2008a) and carcinomas (Galli et al., 2016). Increases in blood basophil counts may also be observed with lymphomatoid granulomatosis (Schultze, 2010; Stockham and Scott, 2008a), and myeloproliferative neoplasms including polycythemia vera, essential thrombocythemia, and primary myelofibrosis (Galli et al., 2016; Stockham and Scott, 2008a).

Other miscellaneous causes of inflammatory increases in blood basophil counts include ulcerative colitis (Juhlin, 1963a), the systemic mast cell disorder urticaria pigmentosa (Asboe-Hansen, 1960), juvenile rheumatoid arthritis (Athreya et al., 1975), and immunological responses causing acute rejection of some tissue grafts (Tikkanen et al., 2001).

12.11.2.5.1.2 Endocrinopathy

Several endocrinopathies have been associated with increases in blood basophil counts. These endocrinopathies include hypothyroidism (myxedema) and diabetes mellitus (Galli et al., 2016; Shelley and Parnes, 1965). It has been suggested that the increases in basophil counts are secondary to hyperlipidemia associated with the endocrinopathies, but supporting mechanistic evidence is scant (Pohlman, 2010; Schultze, 2010).

12.11.2.5.1.3 Neoplasia

Although relatively rare, chronic myelogenous leukemia is frequently associated with increases in blood basophil counts (Spiers et al., 1977), in which the blood basophils have been demonstrated through cytogenetic analysis to arise from the neoplastic clone (Goh and Anderson, 1979). However, there is some suggestion that chronic basophilic leukemia may be a separate process than basophilic chronic myeloid or granulocytic leukemia (Pardanani et al., 2003). Chronic leukemia associated with clonal increases in blood basophil counts also has the potential to undergo blast transformation. Acute basophilic leukemia may also occur but is rare (Duchayne et al., 1999). Other forms of acute myelogenous leukemia may also be associated with increases in basophil counts (Galli et al., 2016).

12.11.2.5.1.4 Xenobiotic-induced

Administration of G-CSF or GM-CSF may cause modest increases in blood basophil counts along with the more pronounced increases in neutrophil and eosinophil counts through stimulation of granulocyte production. Administration of phenylhydrazine as a rat model of hemolysis has been associated with increases in blood leukocyte counts, including basophil counts (Criswell et al., 2000). Perhaps the most common xenobiotic-induced increases in blood basophil counts occur as a hypersensitivity or allergic reaction, and reports have included associated administration of heparin, penicillin, and novobiocin (Schultze, 2010; Shelley, 1963).

12.11.2.5.2 Decreases in basophil counts (basopenia)

The lower reference limit of historical control data in common laboratory species may include basophil counts of 0 cells μL^{-1}. Due to these normally low blood basophil counts in health, decreases in basophil counts may not be detectable or recognizable. However, the following sections list some conditions in which decreased basophil counts have been reported.

12.11.2.5.2.1 Glucocorticoid-induced

Increases in glucocorticoid concentrations, particularly if prolonged, cause decreases in blood basophil counts (Shelley and Parnes, 1965; Juhlin, 1963b; Boseila, 1963). Similar to glucocorticoid-induced decreases in eosinophil counts, there is likely shifting of blood basophils from the circulating to marginating pool. There is also evidence that glucocorticoids cause migration of basophils from circulation into tissues and decrease recirculation from tissue back into blood (Wald et al., 1991). Direct lytic effects on blood or tissue basophils and suppression of basophil production in the bone marrow may also contribute (Boseila, 1963). Myocardial infarction, which has been associated with decreases in blood basophil counts, may be mediated by effects of chronic stress secondary to the ischemic event (Juhlin, 1963c).

12.11.2.5.2.2 Inflammation and hypersensitivity

Severe acute or overwhelming inflammation can lead to a decrease in basophil counts along with decreases in other blood leukocyte counts. A relationship between endotoxemia and decreases in basophil counts has been supported by reductions in blood basophil counts in rabbits administered endotoxin from Salmonella typhi (Goncharova and Krylova, 1967). Also, inflammatory leukocytosis not associated with overwhelming inflammation is often associated with decreases in basophil counts (Galli et al., 2016).

Type I hypersensitivity reactions, which are mediated by rapid IgE release, are commonly associated with decreases in both eosinophil and basophil counts. However, type IV hypersensitivity (cell-mediated) causing histamine release from mast cells may also be associated with decreases in basophil counts. Such hypersensitivity-related decreases in basophil counts can be observed with anaphylaxis and urticaria (Galli et al., 2016; Grattan et al., 1997, 2003; Shelley and Juhlin, 1961).

12.11.2.5.2.3 Endocrinopathy

Hyperthyroidism (thyrotoxicosis) is reported to cause decreases in blood basophil counts (Shelley and Parnes, 1965; Juhlin, 1963c). However, the mechanism of this decrease has not been clearly demonstrated.

12.11.2.5.2.4 Xenobiotic-induced

Exogenous administration of glucocorticoids will result in decreases in blood basophil counts, similar to decreases caused by endogenous glucocorticoids. Also, administration of thyroid hormones or thyroid stimulating hormone to healthy individuals has been reported to cause decreases in blood basophil counts, consistent with the decreases in basophil counts observed with naturally occurring hyperthyroidism (Boseila, 1963).

Acute hypersensitivity reactions to xenobiotics may cause decreases in blood basophil counts. Xenobiotic-induced urticaria, angioedema, and anaphylactic reactions are IgE-mediated type I hypersensitivity reactions, and have been associated with angiotensin-converting enzyme (ACE) inhibitors and various NSAIDs (Roujeau, 2005). Due to the IgE-mediated nature of these reactions, these xenobiotics would have the potential to cause concurrent decreases in blood basophil counts, although IgE-related increases in basophil counts could also occur as described previously. Menthol has also been associated with urticaria and decreases in basophil counts (Papa and Shelley, 1964).

Decreases in blood basophil counts may also occur along with decreases in other leukocyte counts associated with xenobiotic-induced bone marrow suppression and aplastic anemia. As a class, chemotherapeutic agents may cause bone marrow suppression resulting in decreases in multiple leukocyte lineages in blood, including basophils. Xenobiotics implicated in causing aplastic anemia include chloramphenicol, anticonvulsants such as phenytoin and carbamazepine, gold-based compounds, penicillamine, and phenylbutazone (Bloom and Brandt, 2008; Kaufman et al., 1996).

12.11.2.6 Large Unclassified or Other Cells

Some automated hematology analyzers, such as the Siemens Healthcare ADVIA systems, include a “large unclassified cell” (LUC) or “other” cell category in the leukocyte differential. These cells are generally large with no or minimal myeloperoxidase activity, and do not fall within the predefined species’ gating parameters for typically identified leukocyte subtypes. These cells do not represent a distinct cell type, but are most commonly large and/or reactive lymphocytes or monocytes, and increases in LUC counts may be observed with any conditions resulting in increases in blood lymphocyte or monocyte counts. In species where automated basophil counts are not reliable, increases in blood basophil counts may also appear as an increase in LUC counts (Lilliehöök and Tvedten, 2011). Acute leukemia, typified by increases in hematopoietic blast cells in bone marrow and circulation, almost always results in increases in LUC counts. In the presence of high LUC counts, blood smear evaluation should be performed to assess the leukocyte differential and morphologic appearance of the blood leukocytes.

Occasionally there may be mast cells observed in the blood smears of dogs, cats, or laboratory rodents. Mast cells in blood (mastocytemia) typically occur in low in numbers that do not significantly affect the automated leukocyte differential. Such mastocytemia may occur along with an inflammatory response. However, systemic mastocytosis or mast cell leukemia may cause notable increases in blood mast cells, and mast cell neoplasia is the most common cause of circulating mast cells in cats (Skeldon et al., 2010). Blood smear evaluation is required for enumerating mast cells as part of a leukocyte differentiation.

12.11.3 Erythrocytes

The earliest erythrocyte production, or primitive erythropoiesis, occurs in the yolk sac initially and eventually also in the liver during fetal development. Later in fetal development, erythrocyte production switches to predominantly the bone marrow, which is considered definitive erythropoiesis (Harvey, 2012). In neonates, foci of extramedullary hematopoiesis may be observed within the liver histologically. In rats and mice, erythropoiesis within the spleen often significantly contributes to the maintenance of normal blood erythrocyte content. Increases in erythropoiesis in these species may be accompanied by increased extramedullary hematopoiesis in the spleen without appreciable changes in the bone marrow when observed histologically. Increased splenic hematopoiesis in these species may be sufficient to cause detectable changes in organ weight values.

Erythropoiesis is largely under the control of stimulation with erythropoietin (EPO), although stem cell factor (SCF), IL-3, and thrombopoietin (TPO) may also play a role in early erythrocyte differentiation and insulin-like growth factor (IGF)-1 may contribute to later erythropoiesis (Olver, 2010). Erythrocyte differentiation begins with pluripotent hematopoietic stem cells that differentiate into common myeloid progenitor cells, which further differentiate into megakaryocyte–erythrocyte progenitor cells. Under stimulation with EPO, megakaryocyte–erythrocyte progenitors differentiate into the first committed erythroid progenitor, the burst-forming unit erythrocyte (BFU-E), which then differentiates into colony-forming unit erythrocyte (CFU-E) precursors. The next stage in erythroid development is the rubriblast, which is the earliest erythroid progenitor that may be identified with light microscopy. Erythroid cell maturation then progresses through prorubricyte, basophilic rubricyte, polychromatophilic rubricyte, and metarubricyte stages. As these stages progress, the nuclear chromatin becomes more condensed and the nucleus becomes pyknotic, coinciding with increased hemoglobin production and accumulation within the cytoplasm and simultaneously decreased RNA production. At this point, pyknotic nuclei are extruded from the cell to form reticulocytes, which are released from the bone marrow. Proliferative bone marrow erythrocyte pools include rubriblasts through basophilic rubricytes, while maturing bone marrow erythrocyte pools include polychromatophilic rubricytes and reticulocytes.

Reticulocytes may mature into erythrocytes either in the spleen or the blood. Sometimes reticulocytes or mature erythrocytes may contain small remnants or fragments of their nuclei, called Howell-Jolly bodies. These may be observed in low numbers in circulation, but passage blood through the spleen typically results in their removal. Most species have a sinusoidal splenic architecture, but cats have nonsinusoidal splenic architecture and are less efficient at removal of Howell-Jolly bodies, so more circulating erythrocytes with Howell-Jolly bodies may be observed in healthy cats than in other species. Occasionally metarubricytes may also be released from the bone marrow and observed in circulation, although nucleus extrusion typically occurs during splenic passage of these cells. Also, the splenic reticuloendothelial system has a major role in removing damages or senescent erythrocytes from circulation.

The major role of erythrocytes is to carry oxygen, which binds to hemoglobin, from the lungs to tissues. Altered tissue demands for oxygen can increase or decrease the production of EPO and therefore erythropoiesis. In the adult, EPO is produced by the kidney in response to hypoxia.

The circulating blood volume is composed of about 40%–45% erythrocytes (Bloom and Brandt, 2008), but there are usually many noncirculating erythrocytes present within the splenic red pulp. Automated hematology analyzers provide indications of blood erythrocyte counts, blood hemoglobin concentration, and hematocrit, which are collectively indicative of red cell mass. In health, the vast majority of blood erythrocytes are mature erythrocytes, with only a very small proportion of reticulocytes, except in rodents. Rodents normally have mildly greater reticulocyte counts relative to most other common laboratory species due to their higher erythrocyte turnover; gerbils tend to have the shortest erythrocyte lifespans and therefore the highest reticulocyte counts (Zimmerman et al., 2010). Erythrocyte lifespan in circulation is species-dependent. Human erythrocytes have an average lifespan of about 120 days (Thiagarajan and Prchal, 2016), while macaque erythrocytes have a lifespan of approximately 100 days (Provencher Bolliger et al., 2010). Canine and feline erythrocyte lifespans are approximately 100 and 70 days, respectively (Stockham and Scott, 2008b). In contrast, rat erythrocytes have an estimated lifespan of approximately 60 days (Van Putten and Croon, 1958) although there is some strain-related variability (Derelanko, 1987), while mouse erythrocyte lifespans are even shorter, with an estimate of about 41 days (Van Putten and Croon, 1958). Mongolian gerbil erythrocyte lifespans have an estimate of 9–10 days (Zimmerman et al., 2010).

Classic patterns of alterations in red blood cell parameters are summarized in **Table 2**.

12.11.3.1 Increases in Red Cell Mass (Erythrocytosis)

12.11.3.1.1 Relative increases in red cell mass

Relative increases in red cell mass, or hemoconcentration, most commonly occur due to dehydration and splenic contraction. In contrast to the absolute or "true" increases in red cell mass that result from proliferation of erythroid precursors in the bone marrow and/or spleen, relative increases are transient and consist of changes to total blood volume or shifting or noncirculating erythrocytes into circulation resulting in increases in red cell mass, which can rapidly resolve.

12.11.3.1.1.1 Dehydration

Dehydration is a relatively common cause of secondary increases in red cell mass. Dehydration results in depletion of the water content of blood, and a relative increase in the other blood components, including cells (hemoconcentration). Due to the high

Table 2 Classic patterns of alterations in red blood cell components and related endpoints

	Erythrocytosis			*Regenerative anemia*				*Nonregenerative anemia*	
	Relative	*Primary*	*Secondary*	*Blood loss*	*IV hemolysis*	*EV hemolysis*	*Iron deficiency*	*ACD*	*Bone marrow suppression*
RBC	↑	↑ to ↑↑↑	↑ to ↑↑↑	↓ to ↓↓↓	↓ to ↓↓↓	↓ to ↓↓	↓ to ↓↓↓	↓	↓ to ↓↓↓
Retic[a]	-	↑ to ↑↑	↑ to ↑↑	- to ↑↑	- to ↑↑	- to ↑↑	- to ↓[d]	- to ↓	↓ to ↓↓↓
MCV	-	- to ↑	- to ↑	- to ↑	- to ↑	- to ↑	↓	-	-
MCHC	-	- to ↓	- to ↓	- to ↓	↓ to ↑[b]	- to ↓	- to ↓	-	-
Total bilirubin	-	-	-	-	- to ↑↑	↑ to ↑↑	-	-	-
Free Hgb	-	-	-	-	- to ↑↑[c]	-	-	-	-

[a]The magnitude of reticulocyte count increases generally reflects the magnitude of red cell mass decreases; all decreases in red cell mass are initially preregenerative without apparent increases in reticulocyte counts.
[b]When overwhelming intravascular hemolysis causes increases in plasma or serum free hemoglobin, MCHC may be artifactually increased, but MCHC is more commonly decreased due to increases in reticulocyte counts.
[c]Increases in plasma or serum free hemoglobin are only expected to occur with overwhelming intravascular hemolysis.
[d]Chronic iron deficiency anemia is typically nonregenerative with decreases in reticulocyte counts, but increases in reticulocyte counts may be observed following transfusion or iron supplementation.
Patterns described in this table indicate classic or expected changes in red blood cell components and associated endpoints due to these processes. However, duration of these processes, variations in the underlying causes of these conditions, and superimposition of multiple processes may result in differences between expected patterns of leukocyte changes and actual changes observed in an individual.
-, no apparent change; ↑, mild increase; ↑↑, moderate increase; ↑↑↑, marked increase; ↓, mild decrease; ↓↓, moderate decrease; ↓↓↓, marked decrease.
RBC, red blood cells; *Retic*, reticulocytes; *MCV*, mean corpuscular volume; *MCHC*, mean corpuscular hemoglobin concentration; *Hgb*, hemoglobin; *IV*, intravascular; *EV*, extravascular; *ACD*, anemia of chronic disease.

number of erythrocytes present, increases in red cell mass are detectable, whereas increases in leukocyte subtypes are often not observed. These increases in red cell mass are usually observed in conjunction with increases in urea nitrogen and/or creatinine (prerenal azotemia) with concurrent decreases in urine volume and increases in urine specific gravity, as well as proportional increases in albumin and globulin. If evaluating plasma, fibrinogen may also be increased. In nonclinical toxicology studies in rodents, decreased food consumption is often associated with concurrent decreases in water intake, resulting in increases in red cell mass from subclinical or clinical dehydration. Resolution of these secondary increases in red cell mass will occur with adequate rehydration.

12.11.3.1.1.2 Catecholamine-induced

Increases in circulating catecholamine levels in response to fright, excitement, or acute stress can result in increases in red cell mass due to splenic contraction. Noncirculating erythrocytes stored within the red pulp of the spleen are expelled, resulting in increased circulating red cell mass. Such increases in red cell mass are transient and resolve as splenic relaxation occurs following a decline in circulating catecholamine levels. Catecholamine-induced splenic contraction-associated relative increases in red cell mass are most commonly observed at pretest collections in nonclinical toxicology studies utilizing nonhuman primates, dogs, or cats, particularly the first pretest collection if multiple collections are performed. It is typically not observed at subsequent collections as the animal becomes acclimated to the housing, handling/restraint, phlebotomy, and other study-related procedures.

12.11.3.1.1.3 Xenobiotic-induced

Xenobiotic-related causes of relative increases in red cell mass are relatively uncommon, with the exception of dehydration associated with decreased food consumption in rodents utilized in nonclinical toxicology studies, as described earlier. Diuretics, such as furosemide or spironolactone, which result in increased elimination of water into urine, may result in hemoconcentration due to dehydration (Mintzer et al., 2009).

12.11.3.1.2 Secondary increases in red cell mass

Secondary increases in red cell mass are dependent on stimulating factors and are not autonomous, in contrast to primary increases in red cell mass. These secondary increases in red cell mass are most commonly associated with increases in EPO concentrations due to hypoxia, and are therefore considered appropriate. However, hypoxia-independent (inappropriate) mechanisms may also cause secondary increases in red cell mass and are also described later.

12.11.3.1.2.1 Hypoxia-dependent (appropriate)

Increases in EPO production occur in response to hypoxia primarily from the kidney, although there is also some evidence that hypoxia may also stimulate production of EPO by the liver (Rankin et al., 2007). Under normal oxygenation states, hypoxia-inducible factor (HIF) subunits are polyubiquitinated by a von Hipple-Lindau (VHL) tumor suppressor E3 ligase complex, which results in proteosomal degradation of HIF (Jaakkola et al., 2001). Binding of the VHL E3 ligase complex to HIF requires hydroxylation of a proline residue in the VHL protein, a process that requires both oxygen and iron (Ivan et al., 2001). In hypoxic states, HIF is not ubiquitinated and HIF subunits translocate to the nucleus, forming a transcription factor for genes, including the erythropoietin gene. Increases in circulating EPO concentrations as a result of hypoxia cause an

appropriate increase in erythropoiesis and subsequent increase in red cell mass, which should improve delivery of oxygen to tissues.

Sustained tissue hypoxia can result from high altitude residence, cardiac disease causing poor tissue or lung perfusion, or prolonged or chronic pulmonary diseases that impair oxygenation, such as COPD secondary to chronic smoking or obstructive sleep apnea in humans (Prchal, 2016; Stockham and Scott, 2008b). Other conditions that may cause hypoxia-induced increases in red cell mass include mutations leading to hemoglobin with high affinity for oxygen, carboxyhemoglobin formation with heavy smoking, and erythrocyte enzyme deficiencies leading to methemoglobinemia, such as cytochrome b_5 reductase deficiency may also result in hypoxia-induced increases in red cell mass (Prchal, 2016). Erythrocyte enzyme deficiencies resulting in methemoglobinemia have been reported in veterinary species (Stockham and Scott, 2008b), but hemoglobinopathies, conditions characterized by abnormal hemoglobin, have not yet been reported in domestic animals (Randolph et al., 2010).

12.11.3.1.2.2 Hypoxia-independent (inappropriate)

Secondary but hypoxia-independent increases in red cell mass are associated with increases in circulating EPO levels. However, increased EPO in these cases are attributable to autonomous production of EPO rather than hypoxia response. Reported associations include renal diseases, renal or nonrenal neoplasms, or rare dysfunctions of the oxygen sensing pathway.

Nonneoplasic renal diseases associated with increased EPO production include hydronephrosis, renal cysts, and polycystic renal disease (Prchal, 2016). These renal diseases may be associated with local tissue hypoxia (Randolph et al., 2010), but are not associated with systemic hypoxia. Similarly, increases in red cell mass may be observed in humans following renal transplantation (Prchal, 2016).

Autonomous production of EPO by neoplasms has been associated with both benign and malignant tumors. Renal adenoma, renal carcinoma, and sarcoma of the kidney have been reported to cause increases in red cell mass (Ways et al., 1961). Renal lymphoma has also been associated with increases in red cell mass (Durno et al., 2011). Nonrenal tumors with inappropriate EPO production include hepatoma, hamartoma of the liver, leiomyosarcoma, schwannoma, and pheochromocytoma (Stockham and Scott, 2008b; LevGur and Levie, 1995; Muta et al., 1994; Shulkin et al., 1987; Josephs et al., 1961). VHL syndrome, which follows an autosomal dominant pattern of inheritance, may predispose affected people to developing renal or nonrenal neoplasms that can autonomously produce EPO (Prchal, 2016).

There are also rare inherited conditions that cause defects in oxygen sensing pathways described in humans. These include Chuvash polycythemia, which follows an autosomal recessive inheritance pattern, and *EGLN1* gene mutations, which cause a deficiency in proline hydroxylase (Prchal, 2016). High cobalt concentrations may also inhibit the oxygen sensing pathway by preventing binding of the VHL E3 ligase to HIF (Yuan et al., 2003; Schuster et al., 1989).

12.11.3.1.2.3 Endocrinopathies

Increases in red cell mass associated with endocrinopathies are generally mild and do not result in overt clinical signs. Hyperthyroidism causes a sustained increase in tissue demand for oxygen, leading to hypoxia, increased EPO production, and consequent increases in red cell mass (Stockham and Scott, 2008b). Acromegaly, caused by an increase in growth hormone concentrations, has also been associated with increases in red cell mass, particularly in cats (Randolph et al., 2010). Hyperadrenocorticism or adrenal neoplasms that produce androgens or aldosterone may also be associated with increases in red cell mass (Prchal, 2016; Ghio et al., 1981; Mann et al., 1967).

12.11.3.1.2.4 Xenobiotic-induced

Xenobiotic-induced increases in red cell mass are uncommon. Administration of recombinant erythropoietin and anabolic steroids has been reported to cause increases in red cell mass (Mintzer et al., 2009). For example, increases in red cell mass have been associated with testosterone administration (Gardner et al., 1968). Theoretically, excess administration of thyroid hormones could also cause increases in red cell mass.

12.11.3.1.3 Primary increases in red cell mass

Primary, or erythropoietin-independent, increases in red cell mass are associated with myelodysplastic conditions with autonomous production of erythrocytes. Due to the autonomous nature of the proliferations, primary increases in red cell mass are also termed inappropriate as they are not dependent on EPO stimulation. Causes of the more common secondary increases in red cell mass should be excluded prior to the diagnosis of a primary increase in red cell mass. Measurement of EPO concentrations may be useful clinically in people, but assays that quantify EPO are not readily available for most veterinary species. Primary increases in red cell mass are uncommon, and are typically not observed in common laboratory species during nonclinical toxicology studies.

12.11.3.1.3.1 Polycythemia vera

Polycythemia vera is categorized as a chronic myeloproliferative disorder. Neoplastic transformation, from an acquired somatic mutation (Prchal, 2016), of a hematopoietic progenitor cell results in clonal and autonomous expansion of hematopoietic cells, including erythrocytes. Eventually the clonal expansion is sufficient to suppress normal hematopoiesis (Prchal and Prchal, 2016). Increases in red cell mass are the prototypical findings, but concurrent increases in leukocyte and platelet counts that arise from the neoplastic clone are often also be observed in people (Pearson, 2001), although these findings are typically not observed in

dogs or cats (Randolph et al., 2010). The most common causative somatic mutation of polycythemia vera in humans is a mutation of JAK2, a kinase that plays a role in intracellular proliferative signaling (Prchal, 2016). However, forms of polycythemia vera or "idiopathic erythrocytosis" without JAK2 mutations have been observed and associated with mutations in lymphocyte-specific adaptor protein (LNK) that inhibits JAK2 phosphorylation (Lasho et al., 2010). Serum EPO concentrations are expected to be low in patients with polycythemia vera. In domestic dogs and cats, middle-aged female dogs and male cats tend to be most commonly affected (Randolph et al., 2010).

12.11.3.1.3.2 Primary familial and congenital polycythemia

Similar to polycythemia vera, primary familial and congenital polycythemia (PFCP) is also caused by autonomous erythroid proliferation despite low serum EPO. However, PFCP is associated with nonclonal erythroid proliferation from an inherited mutation that has an autosomal dominant pattern of inheritance (Prchal et al., 1985). Identified mutations definitively associated with PFCP result in the truncation of the EPO receptor with a loss of the negative regulatory domain, causing constitutive activity of the signaling pathway promoting erythrocyte proliferation (Prchal and Prchal, 2016).

12.11.3.2 Decreases in Red Cell Mass (Anemia)

Decreases in red cell mass, or anemia, are a relatively common finding in humans and common laboratory species. Decreases in red cell mass are further categorized by concurrent changes in reticulocyte counts, which provide an indication of bone marrow responsiveness and may help to differentiate among possible mechanisms. Decreases in red cell mass with concurrent increases in reticulocyte counts indicate a regenerative erythroid bone marrow response, where normal or low reticulocyte counts may represent a preregenerative, suppressed, or ineffective erythroid response.

12.11.3.2.1 Decreases in red cell mass with increases in reticulocyte counts (regenerative anemia)

Decreases in red cell mass with concurrent increases in reticulocyte counts (reticulocytosis) indicate a regenerative erythroid response by the bone marrow, or by extramedullary hematopoiesis in the spleen of rodents. An increase in reticulocyte count is typically first observed 3–4 days after an acute drop in red cell mass due to bone marrow erythrocyte production and transit time, and peak responses generally occur around 7–14 days depending on the species (Stockham and Scott, 2008b). The regenerative erythroid response is considered appropriate if the increases in reticulocyte counts reflect the magnitude of the decreases in red cell mass; in other words, a mild decrease in red cell mass is expected to result in a mild increase in reticulocyte count, while a moderate to marked decrease in red cell mass should have a concurrent moderate to marked increase in reticulocyte count. The regenerative erythroid response is considered inappropriate if there is an inconsistency between the magnitude of the decrease in red cell mass and the magnitude of the increase in reticulocyte count. For example, a marked decrease in red cell mass with only a mild increase in reticulocyte count a week after the insult would be considered an inappropriate regenerative erythroid response.

Increases in blood reticulocyte counts may be associated with concurrent changes in mean corpuscular volume (MCV; an indicator of erythrocyte size) and mean corpuscular hemoglobin concentration (MCHC; an indicator of erythrocyte hemoglobin content), two red blood cell indices provided by most automated hematology analyzers. Due to the reticulocyte's increased volume relative to mature erythrocytes, increases in blood reticulocyte counts may cause increases in MCV (macrocytosis) and decreases in MCHC (hypochromasia). Regenerative anemias with increases in MCV and decreases in MCHC and/or CHCM may also be classified by these indices as macrocytic, hypochromic anemias. The decrease in MCHC does not necessarily reflect less hemoglobin content per cell, but is a consequence of reduced concentration of hemoglobin due to the larger cytoplasmic volume of reticulocytes. MCHC, which is a calculated endpoint, may be artificially increased when free plasma hemoglobin is present due to intravascular hemolysis. Some automated hematology analyzers also provide the corpuscular mean hemoglobin content (CHCM) which provides a mean of direct measurements of cellular hemoglobin concentration and is therefore resistant to interference from free plasma hemoglobin.

Regenerative erythroid responses with increases in blood reticulocyte counts may be associated with several morphologic findings observed during blood smear evaluation. Most commonly, increases in polychromatophils (polychromasia) are observed with Wright-Giemsa or modified Wright stains. Polychromatophils are erythrocytes that stain blue–purple in color due to the combined effects of blue-staining RNA content typical of reticulocytes and pink-staining hemoglobin. As reticulocytes mature and lose RNA, a visual difference in staining cannot longer be detected between late reticulocytes and mature erythrocytes. However, staining of blood with a vital dye such as New Methylene Blue permits differentiation between aggregate and punctate-type reticulocytes. Polychromasia usually correlates well with increases in reticulocytes in most species, except cats (Stockham and Scott, 2008b). In cats, aggregate-type but not punctate-type reticulocytes correlate with polychromasia and are considered clinically relevant, and differentiation of these two with manual reticulocyte counts should be performed (Harvey, 2012; Stockham and Scott, 2008b). Reticulocytes or erythrocytes with few small, punctate dark blue-gray inclusions may be observed during a regenerative erythroid response. These inclusions contain iron and may be called Pappenheimer bodies or siderotic inclusions. Due to the rapid production and release of erythrocytes during a regenerative erythroid response, there may also be increase in nucleated red blood cells or erythrocytes with Howell-Jolly bodies. Nucleated red blood cells are usually present in low numbers, but if 10 or more are observed per 100 leukocytes, the automated total leukocyte count will be falsely increased

and should be corrected using published equations (Stockham and Scott, 2008a). In some species, particularly cows and sheep, erythrocytes with basophilic stippling may also be observed in circulation during a regenerative erythroid response.

Hemolysis and blood loss are the two main categories of decreases in red cell mass with appropriate increases in reticulocyte counts.

12.11.3.2.1.1 Hemolysis

Destruction of mature erythrocytes is called hemolysis. Hemolysis may occur either intravascularly or extravascularly. With intravascular hemolysis, erythrocyte destruction occurs within the blood and results in hemoglobinemia, or free hemoglobin within plasma. Ghost erythrocytes, or the remnant membranes of erythrocytes that no longer contain cytoplasm or hemoglobin, may be observed with intravascular hemolysis. Consequent hemoglobinuria, or free hemoglobin in the urine, is rare and only occurs in cases of massive intravascular hemolysis that overwhelm the normal pathways that clear free hemoglobin from the blood. In contrast, extravascular hemolysis does not occur within the blood, but rather occurs in the spleen, liver, or bone marrow, where resident macrophages phagocytose erythrocytes and destroy them intracellularly. Extravascular hemolysis does not result in free plasma hemoglobin or hemoglobinuria. Both types of hemolysis may be associated with increases in total bilirubin concentrations where unconjugated (indirect) bilirubin usually exceeds conjugated (direct) bilirubin, and may result in plasma or serum icterus (yellow discoloration) or bilirubinuria (bilirubin present in urine). However, not all cases of hemolysis are clearly either intravascular or extravascular, and both forms of hemolysis may contribute in some conditions.

12.11.3.2.1.1.1 Infectious There are numerous protozoal, bacterial, and viral diseases that can be associated with hemolysis. Mechanisms by which infectious agents cause erythrocyte destruction are varied, and may include direct infection of erythrocytes, elaboration of toxins such as hemolysin, or stimulation of an immune-mediated response against infected cells (Berkowitz, 1991). Several examples of infectious agents that cause hemolytic anemia are discussed later.

Direct infection of erythrocytes with protozoal *Plasmodium* species, the causative agent of malaria that is transmitted by mosquitoes, is a relatively common cause of hemolysis in humans, but may also be observed in nonhuman primates used in nonclinical toxicology studies. Humans are infected by one of five different *Plasmodium* species: *P. falciparum*, *P. vivax*, *P. knowlesi*, *P. malariae*, or *P. ovale*, although only *P. falciparum* and *P. vivax* are commonly associated with severe hemolysis (Lichtman, 2016b). Macaques are most commonly infected with *P. inui* or *P. knowlesi*, although the cynomolgus monkey appears to be more resistant to disease from these infections than the rhesus monkey (Ameri, 2010). Infection with *P. cynomolgi*, *P. fieldi*, or *P. fragile* may also occur in macaques (Magden et al., 2015). Although it is uncommon to include macaques infected with *Plasmodium* species during a nonclinical toxicity study due to current screening practices and pretest evaluations, rare animals with decreases in red cell mass and increases in reticulocyte counts and intraerythrocytic *Plasmodium* organisms have been observed. Rats and mice may be infected with *Plasmodium berghei* (Holloway et al., 1995; Sadun et al., 1965). *Plasmodium berghei* has a specific tropism for reticulocytes rather than mature erythrocytes *Plasmodium* species that infect humans (Car et al., 2006; Cromer et al., 2006). Concurrent increases in reticulocyte counts may occur in early stages or disease or with recrudescence of parasitemia and hemolysis. Hemolysis is associated with clearance of parasitized erythrocytes from circulation predominantly by splenic macrophages (Lichtman, 2016b), although accumulation of hemin, an iron-containing porphyrin, which can directly stimulate apoptotic erythrocyte death (eryptosis) (Gatidis et al., 2009), oxidative damage to erythrocyte membranes (Clark and Hunt, 1983), and increased osmotic fragility (George et al., 1967) may all contribute to hemolysis. However, late-stage infections in humans and rodents have also been associated with inappropriate or decreased reticulocyte counts indicative of suppressed erythropoiesis despite decreases in red cell mass from hemolysis (Lichtman, 2016b; Cromer et al., 2006).

Babesia species are tick-borne protozoal organisms that directly infect erythrocytes in most species, including humans, nonhuman primates, dogs, and cats. *Babesia* species appear as intracellular oval to pyriform organisms. *Babesia microti* and *Babesia divergens* may infect humans in North America and Europe, respectively, and cause moderate hemolytic anemia from intraerythrocytic replication and subsequent erythrocyte lysis (Lichtman, 2016b; Kjemtrup and Conrad, 2000). *Babesia pitheci* has been reported to infect both old and new world monkeys and cause anemia (Magden et al., 2015). *B. canis*, a large babesial species, and *B. gibsoni*, a small babesial species, infect dogs, while cats may be infected by the small babesial organisms *B. felis* and *B. cati* (Stockham and Scott, 2008b; Penzhorn et al., 2004). These organisms are generally not of concern in purpose-bred animals used in nonclinical toxicology studies.

Bartonella bacilliformis in people and the hemotrophic mycoplasmas (hemoplasmas) in dogs and cats (formerly *Haemobartonella* species) and swine (formerly *Eperythrozoon* species) are organisms that parasitize erythrocytes, but these organisms remain extracellular in shallow depressions of the erythrocyte membrane. These organisms are typically round-, rod-, or ring-shaped and may be observed individually or in chains on erythrocyte surfaces. Hemolysis with these organisms may be immune-mediate and associated either with binding of antibodies to parasite antigens or antigens exposed on the erythrocyte secondary to parasite-induced membrane changes (Stockham and Scott, 2008b).

Clostridium perfringens (formerly *Clostridium welchii*) infection in humans is an example of a bacterial cause of hemolysis. During intestinal overgrowth or septicemia, *C. perfringens* type A elaborates an α toxin that has lecithinase C activity, resulting in membrane phospholipid breakdown and release of lysolethicins, which have potent hemolytic capabilities (Lichtman, 2016b; Songer, 1996). *C. perfringens* α toxin release is usually associated with severe intravascular hemolysis with both hemoglobinemia and hemoglobinuria. However, in veterinary species, *C. perfringens*-related hemolysis is typically limited to ruminants and horses (Stockham and Scott, 2008b), and is unlikely to be observed in the common species used in nonclinical toxicology studies.

Infection of humans with *Mycoplasma pneumoniae* has also been associated with hemolytic decreases in red cell mass, although most cases of *M. pneumoniae* infection are asymptomatic. Hemolysis with this organism is attributable to stimulation of autoimmune erythrocyte destruction with agglutination of erythrocytes (Khan and Yassin, 2009).

Several viral organisms in humans have also been reported to cause decreases in red cell mass due to hemolysis. Viral causes of hemolysis are commonly associated with autoimmune mechanisms, and include infection with Epstein-Barr virus (Palanduz et al., 2002), hepatitis A, B, and C viruses (Kanematsu et al., 1996; Chao et al., 2001), cytomegalovirus (Murray et al., 2001), and HIV (Koduri et al., 2002), although HIV infection is also commonly associated with decreases in reticulocyte counts rather than the expected increases secondary to hemolysis, indicative of concurrent suppressed erythropoiesis (Telen et al., 1990).

12.11.3.2.1.1.2 Oxidative Another major cause of decreases in red cell mass due to hemolysis is oxidative damage to erythrocytes. Under normal conditions, ferrous iron (Fe^{2+}) in hemoglobin binds to and dissociates from oxygen as it delivers oxygen from the lungs to the tissues. At times, this binding and dissociation results in the formation of ferric iron (Fe^{3+}) in hemoglobin (methemoglobin) as well as superoxide (O_2^-). Superoxide is a free radical with potent oxidative capacity that may cause cellular damage. Cytochrome b_5-reductase is an intraerythrocytic enzyme that converts methemoglobin back to hemoglobin. Superoxide dismutase converts superoxide to hydrogen peroxide (H_2O_2), which also may produce oxidative damage to cells. Further metabolism of hydrogen peroxide by catalase or glutathione peroxidase protects cells from oxidative damage. These pathways are usually sufficient to address the normal low-level formation of methemoglobin and superoxide, but methemoglobin can increase and impair delivery of oxygen to tissues and superoxide can accumulate and cause oxidative damage if these pathways are overwhelmed or defective.

Oxidative damage to erythrocytes may affect the lipid membranes, cytoskeleton, or hemoglobin. Peroxidation of internal membrane lipids or cytoskeletal components of erythrocytes results in the fusion of portions of the membrane with consequent shifting of the cytoplasm and hemoglobin to one side of the cells. Erythrocytes with this morphologic change are called eccentrocytes. Oxidative damage that causes the formation of eccentrocytes may result in hemolysis due to increased clearance of eccentrocytes by splenic macrophages due to trapping of rigid erythrocytes in splenic sinusoids or spontaneous rupture in blood due to the increased fragility of eccentrocytes (Stockham and Scott, 2008b).

Oxidative damage to exposed cysteine sulfhydryl groups on hemoglobin results in hemoglobin denaturation and decreased solubility (Bloom and Brandt, 2008). Denatured hemoglobin may then precipitate and aggregate within the erythrocyte, forming small, pale-staining round structures that bind to the erythrocyte membrane and tend to protrude from the surface of the erythrocyte. These aggregates of denatured hemoglobin are called Heinz bodies. Cats appear to be particularly sensitive to the formation of Heinz bodies because of an increased number of reactive sulfhydryl groups in hemoglobin relative to other species (Christopher et al., 1990), and may be more rapidly observed on blood smear evaluation due to the nonsinusoidal architecture of the feline spleen that results in decreased clearance of Heinz bodies from circulation. Similar to eccentrocytes, erythrocytes with Heinz bodies may undergo hemolysis due to increased clearance by splenic macrophages following trapping in splenic sinusoids due to decreased erythrocyte deformability and spontaneous rupture due to increased fragility from membrane damage; immune-mediated clearance may also occur and is believed to result from binding of hemochromes to and subsequent redistribution of band 3, an erythrocyte membrane structural protein, which may then be recognized by autologous antibodies (Winterbourn, 1990).

There are many conditions that may cause oxidative damage and result in eccentrocytosis, Heinz body formation, or even both simultaneously. Diabetes mellitus may cause either morphologic change, and diabetic ketoacidosis appears to be associated with an increased susceptibility and incidence of oxidative erythrocyte damage (Desnoyers, 2010; Caldin et al., 2005; Christopher et al., 1995). Inherited deficiencies in erythrocyte glucose-6-phosphate dehydrogenase (G6PD) and flavin adenine dinucleotide (FAD) have also been associated with erythrocyte oxidative damage, eccentrocyte or Heinz body formation, and hemolysis or a predisposition for these events due to the loss of protective antioxidant pathways (Chan et al., 1982; Harvey, 2006). Lymphoma has also been associated with Heinz body formation in cats (Christopher, 1989) and eccentrocytes formation in dogs (Caldin et al., 2005). In dogs and cats, ingestion of *Allium* species, particularly onions, garlic, and Chinese chive, may cause erythrocyte oxidative damage with formation of eccentrocytes and/or Heinz bodies (Caldin et al., 2005; Yamato et al., 2005; Robertson et al., 1998). Ingestion of zinc in dogs (Bexfield et al., 2007) and exposure to skunk musk (Fierro et al., 2013) have also been reported to cause hemolysis due to Heinz body formation.

12.11.3.2.1.1.3 Fragmentation Physical trauma to erythrocytes results in hemolysis due to erythrocyte fragmentation and lysis. Sometimes this type of hemolysis is referred to as microangiopathic hemolytic anemia. Morphologic changes to erythrocytes occur as a result of physical trauma. Schistocytes (also called schizocytes or erythrocyte fragments), keratocytes (also called helmet cells), prekeratocytes (also called blister cells), or even spherocytes or microspherocytes may be observed. Schistocytes are very small, usually irregularly shaped fragments that can break off erythrocytes when physical trauma occurs. Keratocytes have one to two variably sized projections or horns adjacent to a small flattened region of the erythrocyte surface, while prekeratocytes appear to be precursors that have small loops of erythrocyte cytoplasm extending from the surface and surrounding a small hole in the cell. Spherocytes and microspherocytes are spherical cells that appear smaller and have more intensely pink-staining cytoplasm than normal mature erythrocytes. Spherocytes and microspherocytes may be formed during physical trauma as fragments are broken off, resulting in less membrane surface area in the parent erythrocyte surrounding a similar volume (spherocytes) or smaller volume (microspherocytes) as the parent erythrocyte.

The physical trauma to erythrocytes that causes fragmentation or microangiopathic hemolysis may result from consumptive coagulopathies, either local or disseminated (DIC), with fibrin or thrombus formation in the vasculature that impedes the passage of erythrocytes through the vessel lumen, creating both turbulence and physical obstruction of blood flow. Local coagulopathy or DIC may occur secondary to trauma, infections with sepsis, or neoplasia (Baker and Moake, 2016; Toh and Dennis, 2003). Microangiopathic hemolysis due to neoplasia is most commonly associated with malignant rather than benign neoplasms and with metastatic disease or neoplasic emboli rather than primary tumors, with the exception of primary vascular neoplasms (Susano et al., 1994; Kupers et al., 1975; Lohrmann et al., 1973).

Infectious agents may also lead to fragmentation of erythrocytes, and some Leptospirosis interrogans serovars associated with vasculitis (Stockham and Scott, 2008b), *Brucella* species infection (Yaramis et al., 2001), and cutaneous anthrax (Freedman et al., 2002) have been reported to cause microangiopathic hemolysis. In children, fragmentation hemolysis associated with thrombotic microangiopathy may occur with *Shigella dysenteriae* type 1 and some *Escherichia coli* infections (Pisoni et al., 2001). Hemolysis from erythrocyte fragmentation may also occur with HIV infection (Maslo et al., 1997).

Decreases in red cell mass with increases in reticulocyte counts from erythrocyte fragmentation may also occur secondary to cardiac or other conditions that alter hemodynamics and increase turbulent blood flow. For example, subaortic stenosis (Solanki and Sheikh, 1978), intraluminal aortic grafts (Sayar et al., 2006), uncorrected cardiac valvular disease (Marsh and Lewis, 1969), prosthetic valves (Crexells et al., 1972), and hypertrophic obstructive cardiomyopathy (Kubo et al., 2010) have all been reported to cause hemolysis from erythrocyte fragmentation. Increased turbulence associated with hypertension may also cause decreases in red cell mass from fragmentation, and has been associated with pulmonary hypertension (Baker and Moake, 2016) and malignant systemic hypertension (Capelli et al., 1966).

12.11.3.2.1.1.4 Immune-mediated Autoimmune hemolytic anemia (AIHA or AHA) described in humans or immune-mediated hemolytic anemia (IMHA) described in most common laboratory species is a cause of hemolysis, and may be primary or idiopathic, but may also be secondary to conditions such as infections as discussed previously. Primary or idiopathic AIHA/IMHA is discussed here. Primary AIHA has no underlying detectable cause and is an immune-mediated condition that produces antibodies targeting erythrocyte antigens. These antierythrocyte antibodies tend to be very specific for a single erythrocyte antigen in a given case (Packman, 2016). These autoantibodies may be classified as warm antibodies, which are usually IgG, or cold antibodies, which are usually IgM (Stockham and Scott, 2008b).

Immune-mediated AIHA may be associated with erythrocyte morphologic changes that include agglutination and spherocytes. Agglutination may be observed grossly as red speckling along the inside of the specimen tube as blood is gently moved within the tube. If agglutination is present, blood smears may have a "reverse smear" appearance with the densest region of the smear observed at the feathered edge rather than the edge where the drop of blood was initially placed. Microscopically agglutination appears as grape-like clusters of erythrocytes. Spherocytes are erythrocytes that are spherical instead of having the normal biconcave disc shape. While spherocytes appear smaller and stain more intensely pink that unaffected mature erythrocytes, they have the same volume as unaffected erythrocytes. Loss of erythrocyte membrane occurs when macrophages begin to phagocytize antibody-bound erythrocytes, leading to decreased erythrocyte surface area without an appreciable change in volume, forcing erythrocytes to form spheres. Hence, spherocytosis alone will not result in an altered MCV. Of the most common laboratory species, dogs tend to have the most pronounced central pallor of normal mature erythrocytes, making microscopic identification of spherocytes easiest in the dog.

Hemolysis in AIHA is largely attributable to extravascular hemolysis due to phagocytosis of antibody-bound erythrocytes by tissue macrophages. Macrophages or monocytes containing phagocytized erythrocytes may be rarely observed in blood smears of laboratory species with immune-mediated hemolysis. However, antibody-mediated complement activation or increased fragility of spherocytes may result in direct intravascular lysis or rupture of erythrocytes (Packman, 2016). Evaluation of patients for the presence of antierythrocyte antibodies may be performed using the direct antiglobulin test (DAT; also called the Coombs' test) or by flow cytometry.

AIHA has rarely been observed in association with lymphoproliferative neoplasia, such as chronic lymphocytic leukemia. Antierythrocyte antibodies in chronic lymphocytic leukemia are predominantly IgG with few cases of IgM reported (Mauro et al., 2000).

IMHA may occasionally be observed following blood transfusion (Garratty, 2004). This may occur in response to alloantigens, and would not technically be considered autoimmune (Stockham and Scott, 2008b). Posttransfusion immune-mediated hemolysis may also be observed when the host has autoantibodies that bind the donor erythrocytes and cause immune-mediated destruction. However, crossmatching of host and donor erythrocytes and plasma is able to prevent many cases with incompatible transfusion-related AIHA.

AIHA and IMHA commonly have concurrent inflammatory increases in leukocyte subtype counts, characterized mainly by neutrophilia that may or may not have a left shift with cytoplasmic changes indicative of rapid neutropoiesis.

12.11.3.2.1.1.5 Inherited Some phenotypes of sickle cell disease are associated with hemolysis. The mechanism of hemolysis in sickle cell disease is likely multifactorial and not associated with a single pathogenesis. There is evidence for oxidative damage to erythrocytes (Lachant et al., 1983), which may contribute to the hemolysis observed with sickle cell disease. However, hemoglobin polymerization leads to erythrocyte deformation and may lead to decreased flexibility of erythrocytes and veno-occlusive disorders (Bookchin and Lew, 1996). Decreased flexibility or deformability of erythrocytes may contribute directly to increased

cell fragility and rupture or promote clearance of deformed erythrocytes by splenic macrophages, while veno-occlusive disease has the potential to cause decreases in red cell mass through physical trauma and fragmentation. However, there is also evidence that in some severe cases of sickle cell disease there may be an increase in reticulocyte counts that are inappropriate for the magnitude of the decrease in red cell mass, suggesting a concurrent mechanism causing suppressed or ineffective erythropoiesis (Wu et al., 2005; Bookchin and Lew, 1996). Oxidative stress on erythroid precursors may also contribute to ineffective erythropoiesis in some severe cases of sickle cell disease (Fibach and Rachmilewitz, 2008).

Several metabolic defects of erythrocyte metabolism may also be associated with decreases in red cell mass and increases in reticulocyte counts. Deficiencies in erythrocyte pathways of glycolysis may result in decreased ATP concentrations that lead to erythrocyte membrane dysfunctions with shortened erythrocyte lifespan and occasionally hemolysis (Stockham and Scott, 2008b). Pyruvate kinase (PK) is the enzyme that catalyzes the last step in aerobic glycolysis. Deficiencies of PK that result in hemolysis have been reported in humans (Baronciani and Beutler, 1993), dogs including beagles (Harvey et al., 1977; Giger et al., 1991; Prasse et al., 1975), and a few breeds of cats (Kohn and Fumi, 2008). Phosphofructokinase (PFK) catalyzes the rate-limiting step of the glycolysis pathway. Deficiencies in PFK have also been described in humans (Etiemble et al., 1976) and dogs (Giger et al., 1985). Respiratory alkalosis, which may be observed following intense exercise, is associated with acute hemolytic crises in patients with PFK deficiencies (Giger et al., 1985).

The association of inherited G6PD and FAD deficiencies with hemolysis resulting from oxidative damage is discussed earlier. In brief, G6PD and FAD play a role in the antioxidant pathways of erythrocytes. Deficiencies of G6PD and FAD may result in increased oxidative damage to erythrocytes and subsequent hemolysis.

Collectively, the porphyrias are a group of enzymatic defects in the heme synthesis pathway. Porphyrias may be congenital or, more commonly, acquired. In these conditions, the accumulation of porphyrins, the precursors of heme, within erythrocytes leads to hemolysis. The mechanism of hemolysis may be related to lysis of erythrocytes following exposure to light (photolysis) in superficial vasculature, or by direct erythrocyte membrane damage due to the lipid soluble nature of porphyrins or following porphyrin absorption of ultraviolet light and excitation (Phillips and Anderson, 2016; Kaneko, 2008).

12.11.3.2.1.1.6 Neoplastic Various neoplastic conditions may be associated with hemolysis. Malignant metastatic neoplasms or primary vascular neoplasms may result in fragmentation hemolysis by physical trauma to erythrocytes, as previously discussed. However, neoplastic conditions may also rarely be associated with phagocytosis and destruction of erythrocytes, or hemophagocytic syndrome. Hemophagocytic syndromes have been associated with T-cell lymphoma (Gonzalez et al., 1991), NK-cell leukemia (Kobayashi et al., 1996), hemophagocytic histiocytic sarcoma (Moore et al., 2006), and various hematological neoplasias (Majluf-Cruz et al., 1998).

12.11.3.2.1.1.7 Xenobiotic-induced Many xenobiotics are capable of causing hemolysis, and may cause hemolysis through oxidative, fragmentation, or immune-mediated mechanisms. Examples of each are discussed here.

Many of the agents that cause oxidative erythrocyte injury contain aromatic structures that can be metabolized, mostly commonly by cytochrome *P*450, to free radicals (Bradberry, 2003; Edwards and Fuller, 1996), which overwhelm the normal protective antioxidant pathways of erythrocytes leading to both direct erythrocyte oxidative injury and oxidation of hemoglobin sulfhydryl groups resulting in methemoglobin formation. A few specific aromatic compounds that have been associated with free radical formation include dapsone, phenacetin, and anthracyclines such as doxorubicin (Edwards and Fuller, 1996; Coleman et al., 1991; Handa and Sato, 1975; Easley and Condon, 1974). Phenacetin has also been associated with the formation of Heinz bodies (Boelsterli et al., 1983). In dogs and cats, acetaminophen (paracetamol) may be metabolized to a minor reactive metabolite that causes oxidative damage to erythrocytes resulting in hemolysis and the formation of Heinz bodies and/or eccentrocytes, although methemoglobinemia has also been observed in cats (Desnoyers, 2010; Wallace et al., 2002; Mariani and Fulton, 2001; Aronson and Drobatz, 1996). Xenobiotics that cause methemoglobinemia can also cause indirect oxidative damage through the peroxidation activity of methemoglobin itself (Edwards and Fuller, 1996).

In some cases, oxygenated hemoglobin may act as a peroxidase and cause the metabolism of a xenobiotic to a reactive compound that causes erythrocyte oxidative damage and conversion of oxyhemoglobin to methemoglobin. Examples of xenobiotics that cause oxidative damage through this mechanism are phenylhydrazine and primaquine (Edwards and Fuller, 1996). Vitamin K administration in dogs can also cause oxidative erythrocyte damage through this mechanism (Fernandez et al., 1984).

Some chemical agents may cause oxidative damage by directly oxidizing hemoglobin sulfhydryl groups or through direct oxidation of erythrocyte cytoskeletal proteins. Arsine gas, predominantly an environmental toxin, appears to mediate its hemolytic effects through erythrocyte membrane oxidation (Rael et al., 2000), although studies in mice have also demonstrated the formation of Heinz bodies following exposure (Blair et al., 1990), suggesting an oxidative effect on hemoglobin as well.

Many xenobiotics may also cause hemolysis through their association with microangiopathy, most commonly as part of the thrombotic microangiopathy syndrome, which is associated with fragmentation hemolysis and decreases in platelet counts. Drug-induced endothelial injury, including from direct and antibody or immune complex-mediated mechanisms, plays a major role in the pathogenesis of thrombotic microangiopathy (Pisoni et al., 2001). Endothelial damage may be propagated by leukocyte adhesion and release of granule contents or reactive oxygen species, platelet activation and aggregation, and complement activation (Pisoni et al, 2001). Drugs implicated in thrombotic microangiopathy include chemotherapeutic agents include xenobiotics from a wide variety of chemotherapeutic classes. Examples of chemotherapeutics associated with thrombotic microangiopathy include

mitomycin C (Cantrell et al., 1985), cisplatin (Palmisano et al., 1998), estramustine phosphate (Tassinari et al., 1999), gemcitabine (Nackaerts et al., 1998), and daunorubicin (Byrnes et al., 1986). Nonchemotherapeutic agents that have been reported to cause thrombotic microangiopathy include immunomodulators such as cyclosporine and tacrolimus (Katznelson et al., 1994; Trimarchi et al., 1999), simvastatin (McCarthy et al., 1998), and inhibitors of platelet aggregation including ticlopidine and clopidogrel (Bennett et al., 1998, 2000).

Immune-mediated mechanisms of hemolysis have also been reported following exposure to numerous xenobiotics. Xenobiotics may induce antibodies by binding to the erythrocyte membrane and acting as haptens. These antibodies are considered drug-dependent as they only mediate hemolysis when the drug is present. Penicillin is the prototypical xenobiotic that acts as a hapten to generate drug-dependent antibodies, and typically induces an IgG response (Ferner, 2012; Petz et al., 1966). Semisynthetic penicillins, some cephalosporins, and tetracycline have also been reported to cause drug-dependent antibody-mediated hemolysis (Garratty, 2010; Tuffs and Manoharan, 1986; Seldon et al., 1982; Großjohann et al., 2004; Gallagher et al., 1992; Branch et al., 1985; Simpson et al., 1985).

Xenobiotics may also induce the production of antierythrocyte antibodies that mediate hemolysis even when the drug is no longer present, also called drug-independent antibodies or autoantibodies. In this type of hemolysis, xenobiotic exposure stimulates production of an antibody that can bind to native erythrocyte antigens even in the absence of the drug. This type of immune-mediated xenobiotic-induced hemolysis is classically caused by α-methyldopa, and is characterized by predominantly an IgG response (Packman, 2016). However, nucleoside purine analogs such as cladribine and fludarabine have also been associated with hemolysis due to production of autoimmune antibodies (Garratty, 2010; Mintzer et al., 2009; Hamblin, 2006).

A third mechanism by which xenobiotics may cause immune-mediated hemolysis is through a complex interaction of the drug, a drug binding site on erythrocytes, and an antibody. This mechanism is considered the ternary complex mechanism, but has previously, and perhaps less accurately, been called an immune complex or innocent bystander mechanism (Packman, 2016). Quinidine is the prototypical drug that causes hemolysis via this mechanism. Quinidine may be associated with either IgM or IgG antibodies and predominantly causes complement-mediated lysis of erythrocytes or clearance of complement-coated erythrocytes by tissue macrophages (Packman, 2016). Ceftriaxone has also been reported to cause hemolysis through this mechanism (Arndt and Garratty, 2005).

Xenobiotic-induced immune-mediated hemolysis may not be limited to one of the three mechanisms described earlier, and a combination of these mechanisms may occur in some patients. For example, the NSAID diclofenac may cause hemolysis through both drug-dependent and drug-independent mechanisms (Salama et al., 1996). Carboplatin has been reported to cause hemolysis through all three immune-mediated mechanisms (Marani et al., 1996).

Other compounds may cause hemolysis through mechanisms other than oxidative, microangiopathic, or immune-mediated. For example, although the primary effect of lead toxicity is impairment of heme synthesis, lead may also cause hemolysis. The mechanism of lead-induced hemolysis has not been fully determined, but interference with the erythrocyte membrane sodium/potassium transporter may be involved (Bloom and Brandt, 2008). Copper toxicity causes hemolysis as well, possibly through inhibition of many enzymes involved in glycolysis resulting in decreased intracellular ATP (Boulard et al., 1972). Envenomation from multiple animals is reported to cause hemolysis. Envenomation by snakes, such as rattlesnakes and coral snakes, can cause hemolysis through phospholipase A2 activity, which may cause direct hemolysis or liberate hemolysins such as lysolethicin, or through complement-mediated hemolysis (Arce-Bejarano et al., 2014; Tambourgi and van den Berg, 2014; Walton et al., 1997).

12.11.3.2.1.2 Blood loss

12.11.3.2.1.2.1 Hemorrhage Hemorrhage may cause internal or external blood loss. Due to the loss of whole blood during hemorrhage, decreases in red cell mass are usually accompanied by proportionate decreases in albumin and globulin concentrations. The decreases in plasma proteins tend to be less pronounced with internal hemorrhage because the lost proteins may be resorbed in lymph and returned to blood (Stockham and Scott, 2008b). Cases of internal hemorrhage are typically not associated with iron deficiency. However, prolonged external blood loss may cause depletion of total body iron. Iron deficiency anemia is characterized by small erythrocytes with a decrease in MCV and erythrocytes that contain less hemoglobin with a decrease in MCHC, and may be classified as a microcytic, hypochromic anemia. Hemoglobin synthesis plays a role in inhibiting erythrocyte division, and when sufficient iron is not available for heme production, there is loss of the inhibitory effect resulting in more cell divisions and microcytes (Stohlman et al., 1963). Hypochromasia of the erythrocytes is due to the lower than normal hemoglobin content due to decreased production of heme. Morphologic erythrocyte changes that accompany iron deficiency anemia include visual microcytosis and hypochromasia, keratocytes and schistocytes from physical damage to the more fragile erythrocytes, and sometimes codocytes (also called target cells) that have a thin rim of pink-staining hemoglobin and a small central area of hemoglobin with a ring of pallor in between, typical of erythrocytes with less hemoglobin present relative to the amount of membrane. In chronic iron deficiency, increases in reticulocyte counts and microscopic polychromasia may be lower than expected for a regenerative anemia due to loss of RNA during the extended maturation phase of erythrocyte production caused by decreased hemoglobin content (Burkhard et al., 2001).

Direct damage to blood vessels from trauma is a relatively common cause of acute external or internal blood loss. Traumatic rupture of the spleen may also cause significant acute internal blood loss. Decreases in red cell mass due to acute hemorrhage are typically due to dilution of remaining blood from shifting of intracellular fluid to extracellular fluid in an attempt to preserve blood volume and therefore tissue perfusion (Stockham and Scott, 2008b). Dilution of red cell mass may also be observed following administration of intravenous fluids to replace blood volume. A detectable increase in reticulocyte count is expected

3–4 days following the acute event in a patient with normally functioning bone marrow. Damage to blood vessels that results in hemorrhage also may occur secondary to ulcerative or neoplastic conditions. In dogs, rupture of splenic hemangiosarcoma is a common cause of internal blood loss into the abdomen (hemoabdomen). Ulceration of the gastrointestinal system may lead to blood loss into feces, which can be observed as black, tarry feces (melena) if the ulceration occurs in the small intestines or as frank blood if the ulceration occurs in the large intestines.

In humans and nonhuman primate species with true menstrual cycles, including Old World monkeys and great apes (Provencher Bolliger et al., 2010), decreases in red cell mass are uncommon but may be observed from menses-related blood loss. In women, heavy blood loss, abnormal cycling, or uterine neoplasms may lead to sufficient blood loss to cause decreases in red cell mass and potentially even iron deficiency (Van Voorhis, 2009; Goel and Gupta, 2007). In cynomolgus monkeys, decreases in red cell mass have been occasionally observed in females with prolonged menses (Perigard et al., 2016).

Coagulation disorders may also be associated with either internal or external hemorrhage. Primary deficiencies in coagulation factors or von Willebrand factor may be inherited causes of hemorrhage. Deficiencies in coagulation factors that lead to hemorrhage sufficient to cause decreases in red cell mass include hemophilia A (factor VIII deficiency) and hemophilia B (factor IX deficiency); deficiencies in factor XI and von Willebrand factor are usually mild and are often not associated with notable hemorrhage (Bolton-Maggs and Pasi, 2003). Hemorrhage may be secondary to marked decreases in platelet counts from consumptive coagulopathies secondary to infectious or neoplastic processes.

Although not truly hemorrhage, external blood loss can occur from repeated phlebotomy. Decreases in red cell mass may be acutely observed following collection of blood from donors for transfusion, and regular donors have a risk of developing iron deficiency from repeated external blood loss (Cable et al., 2011). Repeated phlebotomy is a common occurrence in nonclinical toxicology studies, particularly in dogs and nonhuman primates, although rats may also occasionally undergo repeated blood collections. Blood is collected through the studies mainly for toxicokinetic or pharmacokinetic analysis, but also for analysis of hematology, coagulation, and clinical chemistry profiles. Decreases in red cell mass with increases in reticulocyte counts of similar magnitude relative to pretest values across all treatment groups, including controls, are a common procedure-related phenomenon in nonclinical toxicology studies and should be distinguished from a true test article-related effect.

12.11.3.2.1.2.2 Parasitism Both external and internal parasites may contribute to blood loss. Hookworms are a major internal parasite associated with chronic blood loss, and may lead to iron deficiency with prolonged infections (Stoltzfus et al., 1997). However, whipworm infection and schistosomiasis may also be associated with blood loss, the latter being associated with blood loss through the urinary system (Farid et al., 1969). Heavy infestation of animals with arthropods that take blood meals, such as ticks, some lice, and fleas, may also cause sufficient blood loss to result in decreases in red cell mass (Stockham and Scott, 2008b).

12.11.3.2.1.2.3 Xenobiotic-induced Xenobiotic-induced blood loss is relatively uncommon but can occur. Classically, hemorrhage into the intestinal tract can result from ulceration associated with chronic NSAID or coxib administration (Laine et al., 2003; Langman et al., 1999; Bjarnason et al., 1987). Also, prolonged or high dose administration of anticoagulants, such as warfarin or heparin, can result in hemorrhage-related decreases in red cell mass (Levine et al., 2001). Ingestion of rodenticides, including brodifacoum chlorophacinone, has been reported to cause marked hemorrhage in humans and many other nonrodent species (Berny et al., 2010; Palmer et al., 1999; Sheafor and Couto, 1999).

Xenobiotic-induced marked decreases in platelet counts may also be associated with hemorrhage and are discussed in more detail later. However, chemotherapeutics that cause bone marrow suppression can be associated with spontaneous or postvenipuncture hemorrhage. Occasional idiopathic decreases in platelet counts have also been observed following xenobiotic administration and are most likely attributable to immune-mediated destruction; some examples of implicated xenobiotics are quinine, trimethoprim–sulfamethoxazole, anticonvulsants such as phenytoin and carbamazepine, unfractionated or low molecular weight heparin, and rituximab (Aster and Bougie, 2007).

12.11.3.2.2 Decreases in red cell mass with "normal" or low reticulocyte counts (nonregenerative anemia)

12.11.3.2.2.1 Preregenerative

Depending on the timing of the insult causing the decreases in red cell mass, reticulocyte counts within reference interval may represent a preregenerative anemia rather than suppressed erythropoiesis. Production of erythrocytes by the bone marrow requires at least 3–4 days, and a peak increase in blood reticulocyte count occurs about 7–14 days following the insult (Stockham and Scott, 2008b). If it an individual with decreased red cell mass and reticulocyte counts that are within the reference interval and it is unclear if the individual has a preregenerative anemia or suppressed erythropoiesis, repeating a CBC several days later may help clarify which process is occurring.

12.11.3.2.2.2 Infectious

Acute Chagas disease, caused by infection with *Trypanosoma cruzi*, has been reported to cause decreases in red cell mass in humans and monkeys (de Titto and Araujo, 1988; Rosner et al., 1988; Seah et al., 1974). In experimentally infected *Cebus paella* monkeys, the acute phase of Chagas disease was reported to cause normocytic, normochromic anemia (Rosner et al., 1988), typical of a nonregenerative anemia. Experimentally infected mice demonstrated bone marrow suppression with decreases in red cell mass as well as decreases in leukocyte and platelet counts (Marcondes et al., 2000). Although rarely encountered in nonclinical toxicology studies,

monkeys held in the southwestern United States may become infected with *T. cruzi* prior to distribution (Magden et al., 2015). During parasitemia, trypomastigotes may be observed in peripheral blood smears.

Viral infections may also cause decreases in red cell mass without concurrent increases in reticulocyte counts. Parvoviruses may cause decreases in red cell mass from direct infection of erythroid precursor resulting in decreased erythrocyte production, as well as decreased erythrocyte lifespans. Parvovirus may result in transient pure red cell aplasia (PRCA) in humans (Van Horn et al., 1986). Cell-mediated suppression of erythropoiesis resulting in PRCA has also been reported with viral hepatitis (Wilson et al., 1980) and Epstein-Barr virus infection (Socinski et al., 1984). Although HIV infection can result in decreases in red cell mass through various mechanisms, direct infection of erythroid precursors appears to contribute to suppressed erythropoiesis (Evans and Scadden, 2000). In cats, a membrane protein of feline leukemia virus has been associated with decreased growth of CFU-E (Wellman et al., 1984). Flavivirus infection, such as dengue, may also result in decreases in red cell mass and reticulocyte counts through bone marrow suppression (La Russa and Innis, 1995).

12.11.3.2.2.3 Chronic disease

Anemia of chronic disease (ACD) is a relatively common cause of anemia, and anemia associated with inflammatory disease is included in ACD. The decreases in red cell mass observed with ACD are generally mild, and are generally normocytic, normochromic, indicating no changes in MCV or MCHC, respectively. ACD may occur through shortening of erythrocyte lifespans, alterations in iron metabolism, a blunted response of erythroid precursors to EPO, and decreased EPO production. Altered erythrocyte lifespans in patients with ACD may be related to increased macrophagic clearance of erythrocytes from circulation through unknown mechanisms (Ganz, 2016). This type of mechanism has been associated with several chronic infections, including tuberculosis and endocarditis (Weiss, 2002).

More commonly, ACD is associated with impaired iron mobilization with low iron concentrations in serum or plasma despite adequate iron stores (Means, 2000). Impaired mobilization of iron results from IL-6 induction of hepcidin that results in sequestration of iron in macrophages and decreased intestinal iron update (Ganz, 2003), IL-1 stimulation of increased synthesis of ferritin which may bind to iron and impair delivery of iron to erythroid precursors (Rogers et al., 1994), and with decreased expression and impaired internalization of the transferrin receptor (Means, 2000). ACD from impaired iron metabolism is associated with numerous inflammatory, infectious, and even neoplastic conditions.

ACD may also cause altered EPO responsiveness or decreased EPO production. Decreased responsiveness of erythroid precursors to EPO is cytokine-mediated, and has been associated with increases in TNFα, IL-1, and interferons (Johnson et al., 1989, 1990; Raefsky et al., 1985) that may commonly be associated with inflammatory conditions. Decreased EPO production may also be cytokine-mediated, and has been reported with increases in TGFβ, TNFα, and IL-1 (Faquin et al., 1992; Jelkmann et al., 1992). However, chronic renal disease may also result directly in impaired EPO production and decreased production of erythrocytes (Sato and Yanagita, 2013).

12.11.3.2.2.4 Immune-mediated

Immune-mediated destruction of erythroid precursors in the bone marrow results in decreases in red cell mass with concurrent decreases in reticulocyte counts. The immune-mediated conditions discussed here may represent a spectrum of disease associated with immune destruction of various stages of erythroid precursors rather than unrelated entities.

12.11.3.2.2.4.1 Autoimmune hemolytic anemia with decreases in reticulocyte counts Autoimmune hemolytic anemia with antibodies that target antigens on mid- to late-stage erythroid precursors ranging from rubricytes to metarubricytes results in AIHA with a decrease in reticulocyte count, which may also be called immune-mediated nonregenerative anemia or precursor-targeted immune-mediated anemia (PIMA). AIHA with reticulocytopenia is generally a normocytic, normochromic anemia. Bone marrow examination may reveal erythroid hyperplasia or maturation arrest (Weiss, 2008) with pyramidal expansion of erythroid precursors at stages earlier than the targeted stage, indicative of ineffective erythropoiesis. This may be less apparent with autoantibodies that recognize more immature stages of erythroid precursors. Bone marrow evaluation may also reveal rubriphagocytosis, or erythroid precursors phagocytized by macrophages. The stage of phagocytized precursor depends on the stage or stages expressing the targeted antigen.

12.11.3.2.2.4.2 Pure red cell aplasia In patients affected by PRCA, there are marked decreases in reticulocyte counts along with variable decreases in red cell mass. Bone marrow examination typically reveals an absence of erythroid precursors (erythroid aplasia) or low numbers of the earliest stages of erythroid precursors (erythroid hypoplasia) (Young, 2016). PRCA in people may be caused by antibodies that bind antigens on the earliest erythroid precursors or even antibodies that bind EPO and prevent EPO-dependent erythropoiesis, but it has also been attributed to clonal T-cell disorders (Stockham and Scott, 2008b). PRCA in dogs has been associated with IgG that inhibit erythropoiesis (Weiss, 1986). PRCA may also be caused by inherited genetic defect in people. Inherited PRCA in people is called Diamond-Blackfan anemia, and often has an autosomal dominant inheritance pattern with defects in genes encoding ribosomal proteins (Young, 2016). Macrocytosis, or increased numbers of large erythrocytes with increases in MCV, may be observed and is consistent with impaired EPO-dependent erythropoiesis (Young, 2016; Ohene-Abuakwa et al., 2005).

12.11.3.2.2.4.3 Aplastic anemia Aplastic anemia is a condition associated with decreases in all cellular blood components (pancytopenia), including decreases in red cell mass with concurrent decreases in reticulocyte counts. Upon examination, the bone marrow classically had severe hypocellularity of hematopoietic cells or an absence of hematopoietic precursors the marrow cavities filled by mostly adipocytes and some stromal elements. Aplastic anemia is thought to be most commonly immune-mediated (Young et al., 2006), and may be frequently associated with cytotoxic T-cells that become autoreactive (Segel and Lichtman, 2016). However, there are also cases of inherited aplastic anemia, most commonly Fanconi anemia associated with genetic mutations that impair DNA repair resulting in pancytopenia developing around 5–10 years of age in people (Segel and Lichtman, 2016). A form of aplastic anemia associated with bone marrow depletion or hypocellularity of hematopoietic tissue and gelatinous transformation of marrow cavity fat has been reported with anorexia nervosa in people (Abella et al., 2002) and with severe food restriction in rats (Moriyama et al., 2008).

12.11.3.2.2.5 Nutritional deficiencies

In addition to aplastic anemia associated with anorexia nervosa and severe food restriction, other nutritional deficiencies have been associated with ineffective erythropoiesis leading to decreases in red cell mass with decreases in reticulocyte counts. Iron deficiency and deficiencies of the B vitamins folate and cobalamin are examples of these nutritional deficiencies. Chronic iron deficiency results in impaired hematopoiesis due to the inability to synthesize sufficient hemoglobin, which may lead to a decrease in reticulocyte production. Deficiencies in folate and cobalamin also cause ineffective erythropoiesis due to defects in DNA synthesis, as discussed with folate and cobalamin deficiencies as a cause of deceases in neutrophil counts. In people, folate and cobalamin deficiencies result in megaloblastic anemia, characterized by larger than normal erythroid precursors in the bone marrow that have more cytoplasm with lower nuclear to cytoplasmic ratios than in normal erythroid precursors and asynchronous cytoplasmic and nuclear maturation (Green, 2016). Megaloblastic erythrocytes may also be observed in circulation, and basophilic stippling or Howell-Jolly bodies may also be observed (Green, 2016). In people, anemia attributable to a deficiency in cobalamin (vitamin B_{12}) may also be called pernicious anemia. In dogs and cats, megaloblastic erythroid cells may be observed in the bone marrow but may not be observed in blood (Stockham and Scott, 2008b).

12.11.3.2.2.6 Endocrinopathy

Several endocrinopathies have also been associated with decreases in red cell mass with decreases in reticulocyte counts, including hypothyroidism, hypoadrenocorticism, and hyperestrogenism. In cases of hypothyroidism, several mechanisms may be contributing to the decreases in red cell mass. Decreased folate or cobalamin concentrations secondary to the hypothyroidism leading to ineffective erythropoiesis, decreased tissue oxygen demand leading to decreased EPO and lower baseline red cell mass, and ACD may contribute to the mild decreases in red cell mass observed with hypothyroidism (Ottesen et al., 1995; Hines et al., 1968; Stockham and Scott, 2008b; Mehmet et al., 2012). Mild decreases in red cell mass without apparent changes in reticulocyte counts have been associated with hypoadrenocorticism. This may be due to a decrease in glucocorticoids, and the loss of the apparent proerythropoietic stimulation of glucocorticoids (Stockham and Scott, 2008b). Hyperestrogenism, which occurs with some ovarian or testicular neoplasms, may result in bone marrow toxicity and suppression of erythropoiesis, particularly in dogs (Sontas et al., 2009).

12.11.3.2.2.7 Neoplasia

Neoplasia may result in suppressed erythropoiesis. This may be due to neoplasia-related inflammation and cytokine release leading to ACD. However, granulocytic leukemia or lymphoproliferative neoplasia involving the bone marrow may result in crowding or effacement of the bone marrow cavities with impaired erythropoiesis that results in decreases in red cell mass with concurrent decreases in reticulocyte counts. Hematopoietic neoplasia involving the erythroid lineage usually results in atypical erythrocyte production that can lead to decreases in red cell mass and reticulocyte counts; however, nucleated erythrocytes with evidence of dysplasia may be observed in blood. Similar to hematopoietic neoplasms that efface the bone marrow, metastatic neoplasia, often carcinomas, may also cause myelophthisis and result in decreased erythropoiesis.

12.11.3.2.2.8 Xenobiotic-induced

There are many xenobiotics that can cause decreases in red cell mass with concurrent decreases in reticulocyte counts. Bone marrow suppression that affects the erythroid lineage is commonly observed with chemotherapeutics in general. For example, agents that are directly cytotoxic to hematopoietic precursors, that inhibit mitotic spindle formation, and antimetabolites that alter folate metabolism may all result in suppression of erythropoiesis. However, development of parvovirus-induced PRCA has been reported as a consequence of chemotherapeutic administration (Song et al., 2002; Rao et al., 1994).

PRCA has occasionally been linked to xenobiotic treatment. A wide variety of xenobiotics from many different classes have been reported to cause PRCA. Examples of xenobiotics reportedly associated with PCRA include sulfonamides, allopurinol, procainamide, gold-containing compounds, rifampin, and chloroquine (Young, 2016; Mintzer et al., 2009). However, causality is often difficult to prove, and most associations are limited to low numbers of case reports. One study evaluated reports of PRCA associated with administration of 30 different xenobiotics, but causality was only attributed to treatment with azathioprine, isoniazid, and phenytoin (Thompson and Gales, 1996). PRCA due to the development of anti-EPO antibodies may follow the administration

of recombinant EPO in humans (Casadevall et al., 2002) and EPO gene therapy in monkeys (Gao et al., 2004). Administration of recombinant EPO to dogs has also led to the development of anti-EPO antibodies and PRCA (Randolph et al., 2004).

Aplastic anemia has also been linked to administration of xenobiotics. Classically, chloramphenicol is reported to sporadically cause aplastic anemia (Segel and Lichtman, 2016). However, antithyroid compounds, sulfonamides including trimethoprim sulfamethoxazole, beta-lactams, the diuretic furosemide, gold-containing compounds, penicillamine, and anticonvulsants including carbamazepine and phenacetin have all been reported in association with aplastic anemia (Mintzer et al., 2009; Kaufman et al., 1996). Aplastic anemia has also been attributed to environmental or occupational exposure to benzene (Smith, 1996). In a case of aplastic anemia in a dog, griseofulvin administration was suspected to be the cause of the aplastic anemia (Brazzell and Weiss, 2006).

Decreases in red cell mass with concurrent decreases in reticulocyte counts have occurred with prolonged or repeated high dose administration of G-CSF or GM-CSF-based xenobiotics in nonclinical toxicology studies, particularly in rodents. Impaired erythropoiesis in these cases occurs due to the massive expansion of myeloid precursors within the bone marrow. Extreme myeloid hyperplasia with continued stimulation results in overcrowding of the marrow cavities with less physical space available for erythroid production.

12.11.4 Platelets

The production of platelets from megakaryocytes (thrombopoiesis) and the production of megakaryocytes (megakaryopoiesis) occur mainly in the bone marrow of adult animals. Common myeloid progenitors differentiate into megakaryocyte-erythroid progenitors. Further differentiation results in formation of the earliest committed megakaryocytic cell, the burst-forming unit-megakaryocyte (BFU-Mk), which further differentiates into the colony-forming unit-megakaryocyte (CFU-Mk). Subsequent stages of differentiation are megakaryoblasts, followed by promegakaryocytes, then megakaryocytes. These latter stages may be recognized during light microscopic evaluation of bone marrow. Extensions of megakaryocyte cytoplasm (proplatelets) enter sinuses, or the microvasculature of the bone marrow. Within sinuses, these proplatelets are detached from the megakaryocyte by the shear forces of blood, after which they are further fragmented into platelets (Harvey, 2012; Junt et al., 2007). Thrombopoiesis, as well as megakaryopoiesis, is predominantly stimulated by TPO. However, SCF, stromal cell-derived factor 1 (SDF-1), IL-3, G-CSF, and GM-CSF may all contributed to platelet production (Boudreaux, 2010).

There are large species-based variations in platelet counts in health; rodents generally have the highest platelet counts of the common laboratory species, which may exceed 1,000,000 platelets μL^{-1}, while nonhuman primates and dogs generally have lower but highly variable platelet counts. The circulating lifespan of platelets is approximately 5–9 days, and up to 30% of circulating platelets may be transiently contained by the spleen (Russell, 2010). Clearance of senescent platelets from circulation is mainly due to phagocytosis by splenic macrophages.

12.11.4.1 Increases in Platelet Counts (Thrombocytosis)

12.11.4.1.1 Catecholamine-induced

Increases in circulating catecholamine concentrations, such as epinephrine, from fright or excitement can result in mobilization of platelets from the spleen into circulation, usually through splenic contraction. The increases in platelet counts from catecholamine-induced redistribution are generally transient and resolve with splenic relaxation and decreases in catecholamine concentrations back to basal levels. Strenuous exercise may also cause redistribution of splenic platelets due to α-adrenergic stimulation, resulting in increases in blood platelet counts (Chamberlain et al., 1990).

12.11.4.1.2 Inflammation or reactive

Inflammatory or reactive increases in platelet counts are typically secondary to a process that causes general bone marrow stimulation, resulting in increased circulating TPO and therefore increased thrombopoiesis. These increases in platelets are not clonal.

Increases in IL-6 may occur as the result of inflammatory or immune stimulation of myriad etiologies, but may also increase as part of a paraneoplastic syndrome in association with malignant neoplasia of nonhemic origin, including renal cell carcinoma, ovarian neoplasia, primary lung cancer, and gastrointestinal neoplasia (Blay et al., 1993; Stone et al., 2012; Yanagawa et al., 1995; Kabir et al., 1995; Lin et al., 2014). Increased IL-6 concentrations have been demonstrated to increase liver production of TPO (Kaser et al., 2001), which stimulates thrombopoiesis with consequent increases in blood platelet counts.

Iron deficiency with decreases in red cell mass has been associated with reactive increases in platelet counts in many, but not all, cases (Stockham and Scott, 2008c). The mechanism of the increases in platelet counts is unclear (Dan, 2005). Some studies have demonstrated that there are no detectable increases in TPO or IL-6 in iron deficiency anemia (Akan et al., 2000), and that although EPO is increased with iron deficiency anemia, cross-reactivity of TPO and EPO does not explain the reactive thrombocytosis (Geddis and Kaushansky, 2003).

Transient increases in blood platelet counts have been reported following splenectomy. Splenectomy has been associated with increases in circulating TPO levels (Ichikawa et al., 1998), resulting in increased platelet product ion and the observed increases in platelet counts.

Increases in TPO also occur during instances of decreases in platelet counts, such as observed with immune-mediated platelet destruction, bone marrow suppression, or blood loss, as discussed later. Following resolution of the cause of decreased blood platelet counts, TPO stimulation of increased production may cause a transient increases in blood platelet counts, or rebound thrombocytosis, prior to normalization of platelet counts (Stockham and Scott, 2008c).

12.11.4.1.3 Neoplastic

Neoplastic processes that cause primary (nonreactive) increases in blood platelet counts are characterized by clonal expansions of megakaryocytes and therefore platelets. Hemic neoplasia that may result in clonal increases in platelet counts include acute megakaryoblastic leukemia and chronic myeloproliferative disease such as primary or essential thrombocythemia. However, clonal increases in platelet counts are associated with increases in TPO greater than increases in TPO observed with reactive thrombocytosis, so TPO-mediated thrombopoiesis may also play a role in clonal increases in platelet counts (Wang et al., 1998).

In acute megakaryoblastic leukemia (AML M7), bone marrow contains $\geq$30% megakaryoblasts, and many cells of the megakaryocytic lineage have cytoplasmic blebs (Gassmann and Löffler, 1995). Myelofibrosis is frequently also observed (Tallman et al., 2000). Acute megakaryoblastic leukemia has been associated with increases in platelet counts, but decreases in platelet counts have also been described (Stockham and Scott, 2008c). A majority of cases of acute megakaryoblastic leukemia in both adults and children are associated with chromosomal abnormalities, often chromosomal translocations (Duchayne et al., 2003; Lion et al., 1992).

Primary thrombocythemia has been associated with marked increases in platelet counts, but bone marrow megakaryoblasts are $<$30% in contrast to acute megakaryoblastic leukemia. Evidence of platelet dysplasia may be observed on microscopic evaluation of blood smears; these morphologic changes may include hypogranular platelets, large platelets associated with an increase in mean platelet volume (MPV), or pleomorphic platelets (Stockham and Scott, 2008c). Similar to polycythemia vera, primary thrombocythemia has been associated with activating mutations in JAK2 (Levine et al., 2005). However, primary thrombocythemia may also be associated with mutations in the TPO receptor gene, *MPL*, and the calreticulin gene, *CALR* (Beer and Green, 2016).

12.11.4.1.4 Xenobiotic-induced

Administration of exogenous catecholamines has been reported to cause increases in blood platelet counts, and the mechanism is primarily from splenic contraction and a transient increased in platelet counts. However, platelet pools from pulmonary circulation may also contribute. For example, administration of epinephrine to dogs resulted in dose-responsive increases in blood platelet counts that were believed to be the result of adrenaline-induced mobilization of platelets from pulmonary circulation into peripheral blood (Bierman et al., 1952).

Increases in platelet counts associated with xenobiotic administration are most commonly reactive, and associated with increases in IL-6 and/or TPO. Any xenobiotic that can result in an inflammatory stimulus may result in reactive increases in platelet counts, and examples of such xenobiotics are discussed in more detail in previous sections. Resolution of xenobiotic-induced bone marrow suppression may cause a transient rebound increase in platelet counts due to increased TPO and thrombopoiesis secondary to the xenobiotic-induced decreases in platelet counts.

G-CSF and GM-CSF-based xenobiotic administration has been associated with increases in blood platelet counts from general bone marrow stimulation. These changes may be due to proliferation of myeloid precursors including common precursors that may then differentiate into megakaryocytes.

Xenobiotic-induced increases in platelet counts have also been attributed to treatment with vinca alkaloids or miconazole, and are believed to be attributable to the ability of these xenobiotics to stimulate increased megakaryocyte production within the bone marrow (Frye and Thompson, 1993). While increases in platelet counts have been reported with treatment with several antibiotic classes, including some cephalosporins, β-lactams, and penicillin, causality is difficult to prove in these cases and a reactive thrombocytosis from inflammation associated with the infectious process being treated must be considered (Frye and Thompson, 1993).

12.11.4.2 Decreases in Platelet Counts (Thrombocytopenia)

Marked decreases in platelet counts are a clinical concern because they may be associated with spontaneous bleeding. Animals are typically considered at risk for spontaneous bleeding when platelet counts are $<$50,000 μL^{-1} and at a significantly greater risk for spontaneous bleeding with $<$10,000 platelets μL^{-1} (Russell, 2010), although hemorrhage with platelet counts $<$50,000 μL^{-1} may also occur following surgery or trauma, including venipuncture routinely performed during nonclinical toxicology studies. Minimal to moderate decreases in platelet counts are typically not associated with hemorrhage, and are not a major clinical concern unless there is a concurrent platelet functional defect. Increased MPV is an indicator of increased average platelet size, and may be associated with rapid or increased thrombopoiesis and indicative of a bone marrow response to decreases in platelet counts.

12.11.4.2.1 Relative

Relative decreases in platelet counts occur when circulating platelet mass is not changed, but platelet clumping, redistribution, or hemodilution alter automated or estimated platelet counts. False decreases in platelet counts (pseudo-thrombocytopenia) due to platelet clumping are often present in rats, mice, and cats, although it may be observed in any species. MPV may be increased with the presence of platelet clumps, and microscopic blood smear review should be performed in all cases of decreased platelet counts

to assess for the presence of platelet clumps; increases in MPV in instances of platelet clumping do not reflect a bone marrow response because a true decrease in platelet count is not present to stimulate thrombopoiesis. Resampling of blood with a clean venipuncture and use of sodium citrate as the anticoagulant may help reduce platelet clumps and result in a more accurate automated platelet count.

Redistribution or sequestration of circulating platelets may cause decreases in platelet counts, which may be transient. Platelet redistribution may be observed with splenomegaly, hypersplenism, or with severe hypothermia; total platelet mass is unaffected in these cases and typically does not result in increased TPO or platelet production (Stockham and Scott, 2008c).

Hemodilution may occur following administration of intravenous fluids or massive transfusion, and is expected to decrease all blood components to variable degrees, with the exception of any transfused blood components. Decreases in platelets from hemodilution are usually mild and may not be detected if platelet counts remain within reference intervals or historical control ranges. These changes are generally transient and resolve with redistribution of intravascular (extracellular) fluid into intracellular fluid compartments or elimination of excess fluid.

12.11.4.2.2 Loss

Acute, severe loss of whole blood may result in decrease in all blood components, including platelets. Blood loss of significant magnitude to cause decreases in blood platelet counts may occur following trauma, splenic rupture from trauma or neoplasia, or from uncontrolled bleeding associated with coagulopathies, such as hemophilia A or B. However, consumption of platelets at the site(s) of hemorrhage may also contribute to the decreases in platelet counts observed with blood loss.

12.11.4.2.3 Decreased survival

Decreased platelet survival may be due to increased platelet destruction and/or consumption, and is a relatively common cause of decreases in blood platelet counts.

Destruction of platelets is commonly associated with immune-mediated mechanisms, including immune-mediated thrombocytopenia (IMT) and immune-mediated thrombocytopenic purpura (ITP) in humans. Most autoantibodies that cause platelet destruction are of the IgG class, although cases with IgM or IgA antiplatelet antibodies have also been reported (Diz-Küçükkaya and López, 2016). Antiplatelet antibodies may directly target platelet antigens, such as the glycoprotein integrin $\alpha_{IIb}\beta_{3(GPIIb\text{-}IIIa)}$ that plays a major role in platelet aggregation (He et al., 1994), antigens exposed or formed on platelet surfaces by infectious agents, or infectious agent-targeted antibodies that cross-react with normally expressed platelet surface antigens or membrane components such as phospholipid (Stockham and Scott, 2008c). Antibody-bound platelets may be phagocytosed and destroyed by splenic, bone marrow, or hepatic macrophages, or may be associated with complement-mediated destruction or stimulation of phagocytosis (Diz-Küçükkaya and López, 2016; Russell, 2010). Production of antiplatelet antibodies may be unassociated with an underlying disease condition (idiopathic or primary) and is usually presumed to be an autoimmune process, or may be secondary to infectious or neoplastic processes (Stockham and Scott, 2008c). In sexually mature Göttingen minipigs, spontaneous thrombocytopenic purpura has been described and is believed to be caused immune-mediated platelet destruction (Carrasco et al., 2003). ITP secondary to infectious disease has been reported to occur with various bacterial and viral etiologies, including *Helicobacter pylori*, many rickettsial diseases, HIV, cytomegalovirus, and hepatitis B and C viruses (Diz-Küçükkaya and López, 2016; Russell, 2010; Cines et al., 2009). Lymphoproliferative neoplasia is a relatively common cause of ITP, and has been associated with chronic lymphocytic leukemia, Hodgkin lymphoma, and leukemia of large granular T-lymphocytes (Cines et al., 2009). Connective tissue diseases such as SLE have also been associated with ITP (Cines et al., 2009).

Decreases in platelet counts due to platelet activation and consumption are commonly associated with local or disseminated consumptive coagulopathies. Localized consumptive coagulopathy may be associated with sites of hemorrhage, microangiopathy or thrombosis, or vascular neoplasia such as hemangiosarcoma (Stockham and Scott, 2008c). Thrombosis associated with chronic catheterization may be a cause of localized platelet consumption. DIC may be associated with infectious agents, particularly bacterial infections that cause septicemia or endotoxemia, hepatic disease, a variety of neoplastic diseases, and pancreatitis (Stockham and Scott, 2008c). Activation and consumption of platelets associated with decreases in platelet counts has also been observed with vasculitis and conditions associated with turbulent blood flow such as endocarditis, cardiac valvular disease, following cardiac surgery, and with arterial disease that alter or damage endothelial cells (Russell, 2010; Selleng et al., 2010; Stockham and Scott, 2008c; Gregg and Goldschmidt-Clermont, 2003). Hemolytic uremic syndrome (HUS) is a cause of thrombotic microangiopathy that may be associated with infection with *Shigella dysenteriae* or *Escherichia coli* (Russell, 2010).

12.11.4.2.4 Decreased production

Bone marrow suppression that involves the megakaryocytic lineage causes decreases in platelet production and therefore decreases in platelet counts. Bone marrow suppression may occur in association with infectious agents that can directly infect hematopoietic precursors such as immunodeficiency viruses in various species, parvoviruses, distemper virus in dogs, and feline leukemia virus in cats (Russell, 2010; Stockham and Scott, 2008c; Scaradavou, 2002). Chronic ehrlichiosis in dogs has also been reported to cause bone marrow hypoplasia, although the mechanism remains unclear (Stockham and Scott, 2008c). However, decreases in platelet counts attributable to infectious agents may have contributions from mechanisms other than bone marrow suppression, such as

peripheral consumption or immune-mediated destruction. Hyperestrogenism associated with testicular or ovarian neoplasia may also cause general bone marrow suppression, and dogs appear to be particularly sensitive to this effect (Sontas et al., 2009).

Causes of decreased bone marrow megakaryocytes with decreases in blood platelet counts have also been associated with mechanisms that are likely immune-mediated. Aplastic anemia, which is most commonly caused by immune-mediated mechanisms (Young et al., 2006), is associated with generalized bone marrow hypoplasia involving all hematopoietic lineages, including megakaryocytes. Amegakaryocytic thrombocytopenic purpura in humans may be inherited or acquired, and acquired forms commonly occur through immune-mediated mechanisms (Diz-Küçükkaya and López, 2016). Inherited or congenital forms cause marked decreases in blood platelet counts with an absence of megakaryocytes in the bone marrow. This form is frequently associated with mutations in the TPO receptor gene, *MPL*, and may progress to aplastic anemia (Germeshausen et al., 2006; Van Den Oudenrijn et al., 2000). Acquired amegakaryocytic thrombocytopenic purpura is likely associated with immune-mediated decreases in bone marrow megakaryocytes (Tristano, 2005), and antibodies that bind TPO or the TPO receptor have also been reported to cause acquired amegakaryocytic thrombocytopenic purpura (Shiozaki et al., 2000; Katsumata et al., 2003).

Generalized bone marrow disease, such as replacement of normal bone marrow hematopoietic tissue with hemic or nonhemic neoplasia, severe granulomatous inflammation effacing normal bone marrow tissue, myelofibrosis, or bone marrow necrosis, will result in decreases in megakaryocytes and thrombopoiesis with subsequent decreases in blood platelet counts (Russell, 2010; Stockham and Scott, 2008c).

12.11.4.2.5 Xenobiotic-induced

Blood loss associated with xenobiotics has been reported with coagulopathies due to abnormal vitamin K recycling and function of vitamin K-dependent coagulation factors. Drugs implicated in abnormal vitamin K synthesis include warfarin, rodenticides such as brodifacoum, and some broad-spectrum antibiotics (Bloom and Brandt, 2008). With severe, acute loss of whole blood, all blood components, including platelets, will be decreased. If other mechanisms of xenobiotic-induced decreases in platelet counts, as discussed later, cause severe enough decreases in blood platelet counts (e.g., $<50{,}000$ platelets μL^{-1}), then blood loss could be secondary to the decreases in platelets.

Thrombotic microangiopathy syndrome, which may include thrombotic thrombocytopenic purpura (TTP) and HUS, may cause peripheral consumption and/or destruction of platelets with the development of decreases in platelet counts. Xenobiotic-induced endothelial injury leads to platelet activation and aggregation (Pisoni et al., 2001). Several chemotherapeutic agents associated with thrombotic microangiopathy syndrome include mitomycin C (Cantrell et al., 1985), cisplatin (Palmisano et al., 1998), estramustine phosphate (Tassinari et al., 1999), gemcitabine (Nackaerts et al., 1998), and daunorubicin (Byrnes et al., 1986). Nonchemotherapeutic agents, including immunomodulators such as cyclosporine and tacrolimus (Katznelson et al., 1994; Trimarchi et al., 1999), simvastatin (McCarthy et al., 1998), and inhibitors of platelet aggregation including ticlopidine and clopidogrel (Bennett et al., 1998, 2000), have also been associated with thrombotic microangiopathy syndrome.

Immune-mediated destruction of platelets is a relatively common cause of xenobiotic-induced decreases in blood platelet counts. Antiplatelet antibodies may be formed through various mechanisms. Penicillin and some cephalosporins cause platelet destruction through hapten-type or drug-dependent antibody production, while quinidine and some NSAIDS can cause immune-mediated platelet destruction through the formation of antibodies that only bind platelets when the soluble drug is present (Aster and Bougie, 2007). Some drugs that inhibit the platelet glycoprotein $\alpha_{IIb}\beta_{3(GPIIb\text{-}IIIa)}$ may lead to platelet expression of a new antigen due to conformation changes to the glycoprotein complexes that can then be bound by antibodies; such drugs include tirofiban, roxifiban, and eptifibatide (Aster and Bougie, 2007; Aster, 2005). Abciximab has been reported to cause drug-specific antibodies that cause decreases in platelet counts (Aster, 2005). Xenobiotic-induced production of autoantibodies that bind platelets, or drug-independent antibodies, has been attributed to procainamide and gold-based compound administration (Aster and Bougie, 2007). In humans, immune-complex type antibodies are classically associated with heparin therapy, and are due to the interaction of heparin, a platelet granule component, and platelet factor 4 (PF4) (Aster and Bougie, 2007; Visentin and Liu, 2007; Arepally and Ortel, 2006).

Bone marrow suppression is caused by numerous xenobiotics, most notably chemotherapeutic agents. Chemotherapeutic agents associated with bone marrow suppression include compounds from many classes, including alkylating agents such as busulfan, antimetabolites that impair DNA synthesis such as methotrexate and mercaptopurine, antibiotics such as doxorubicin, and mitotic spindle inhibitors such as the vinca alkaloids vinblastine and vincristine (Carey, 2003; Weiss, 2010; Stockham and Scott, 2008c). Idiosyncratic myelosuppression may also occur with numerous nonchemotherapeutic xenobiotics, and has been reported with antithyroid drugs such as methimazole, anticonvulsants including felbamate and carbamazepine, antipsychotic agents such as clozapine, cardiovascular drugs such as methyldopa and captopril, antibiotics including trimethoprim–sulfamethoxazole and chloroquine, and other xenobiotic agents including gold-based compounds, diclofenac, and allopurinol (Carey, 2003).

Xenobiotic-induced aplastic anemia is most commonly associated with immune-mediated destruction of uncommitted or early hematopoietic stem cells, although direct cytotoxicity, such as with chemotherapeutic agents, may also lead to aplastic anemia. Several xenobiotics, including chloramphenicol, anticonvulsants such as phenytoin and carbamazepine, gold-based compounds, and phenylbutazone have been associated with aplastic anemia (Bloom and Brandt, 2008).

Miscellaneous causes of xenobiotic-induced decreases in blood platelet counts include nonimmune-mediated destruction reported to occur with desmopressin (Bloom and Brandt, 2008) and idiosyncratic reactions associated with CARPA (Patkó and Szebeni, 2015) or administration of some antisense oligonucleotides (Frazier, 2015). Idiosyncratic decreases in platelet counts have also been reported as an off-target effect of human monoclonal antibodies during nonclinical toxicology studies (Everds et al., 2013b).

12.11.5 Conclusions

There are numerous causes of direct alterations in blood components, including an increasing number of xenobiotics. Alterations in the bone marrow hematopoietic systemic either independent of xenobiotics or induced by xenobiotics may also result in changes in peripheral blood cell counts. Many of the mechanisms of alterations in blood cell counts that are not related to xenobiotic administration have considerable overlap with the mechanisms through which xenobiotics cause changes, and understanding these mechanisms and their association with individual compounds or drug classes may help elucidate potential pathways through which novel xenobiotics cause alterations in blood components.

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