

Review

Toxins—Useful Biochemical Tools for Leukocyte Research

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Abstract: Leukocytes are a heterogeneous group of cells that display differences in anatomic localization, cell surface phenotype, and function. The different subtypes include e.g., granulocytes, monocytes, dendritic cells, T cells, B cells and NK cells. These different cell types represent the cellular component of innate and adaptive immunity. Using certain toxins such as pertussis toxin, cholera toxin or clostridium difficile toxin, the regulatory functions of $G\alpha_i$, $G\alpha_s$ and small GTPases of the Rho family in leukocytes have been reported. A summary of these reports is discussed in this review.

Keywords: toxins; leukocytes; signal transduction

1. Introduction

Leukocytes are a heterogeneous group of cells that display differences in anatomic localization, cell surface phenotype, and function. They originate from CD34 bone marrow stem cells and progenitors of the different subtypes in the bone marrow. Finally, these progenitors or precursors circulate in the blood or are seeded *via* the bloodstream to the tissues where they give rise to highly specialized cells. The different subtypes include e.g., granulocytes (neutrophils, eosinophils, basophils), monocytes, dendritic cells, T cells, B cells and NK cells (Figure 1). These different cell types represent the cellular component of innate and adaptive immunity, protecting the body against invaders and functioning in tumor surveillance. Neutrophils, eosinophils and monocytes are cellular components of innate immunity and are involved in first line of defense against bacteria and helminth infections. Dendritic cells have the ability to take up antigens and readily degrade them in endocytic vesicles to produce antigenic peptides capable of binding major histocompatibility complex (MHC) class II. In response to

danger signals (*i.e.*, tissue damage, pathogen-derived products, or inflammatory cytokines), dendritic cells migrate to lymphoid organs where they interact with CD4⁺ T cells to initiate specific immune responses. CD4⁺ T cells are involved in the development and clonal expansion of effector $\alpha\beta$ T (e.g., CD8⁺ cytotoxic T cells) or B cells. NK cells attack cells that are missing "self" markers of MHC class I without prior activation: therefore, they play a major role in the rejection of tumors and cells infected by viruses. In contrast to $\alpha\beta$ T cells, $\gamma\delta$ T cells do not require antigen processing and MHC presentation of peptide epitopes for recognition of antigens. They are involved in defense against bacteria and in tumor surveillance. In order to fulfill all these different activities, the various subtypes of leukocytes perform highly specialized effector functions, summarized in Table 1.

Figure 1. Differentiation diagram of CD34 bone marrow stem cells and progenitors into the different leukocytes subtypes.

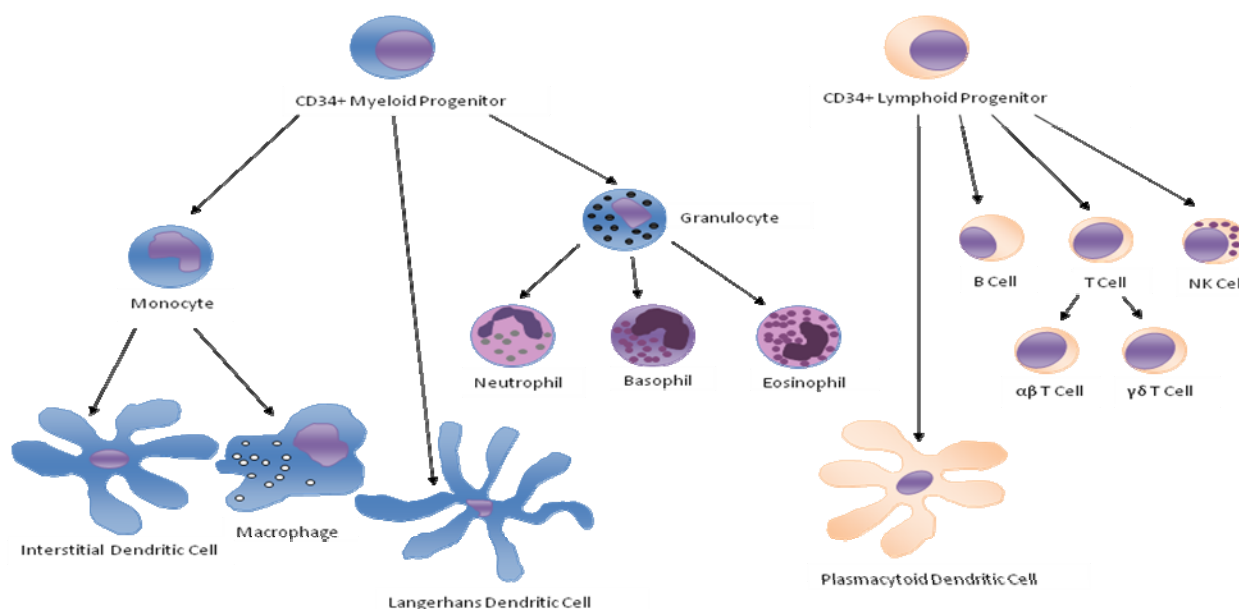


Table 1. The main specific functions of the leukocyte subtypes.

Cell Type	Main functions
Monocytes	Monocytes ingest antibodies or complement opsonized material by phagocytosis [1]. They are able to produce reactive oxygen species. Once activated, monocytes can present antigens to T cells and secrete cytokines such as IL-1, TNF- α , IL-6 and INF- α/β . They can express cell-adhesion molecules and migrate to inflammation sites by chemotaxis .
Neutrophils	Neutrophils can express cell-adhesion molecules that allow diapedesis [2–4] and they can move to a site of infection or inflammation through a process called chemotaxis [5]. They can directly attack micro-organisms by phagocytosis [6] and are able to produce reactive oxygen species [7], release soluble anti-microbials and granule proteins (degranulation), and generate neutrophil extracellular traps (NETs) [8].
B Cells	B cells produce antibodies , which assist in the destruction of microbes by binding to them and making them easier targets for phagocytes and activation of the complement system, and eventually develop into memory B cells after activation by antigen interaction.

Table 1. Cont.

$\alpha\beta$ T Cells	T cells play a central role in cell-mediated immunity. There are several subgroups: T cells that express the CD4 protein on their surface are called T-helper cells because they assist B cells and cytotoxic T cells. T-helper cells recognize peptide antigens associated with MHC-class II on the surface of antigen presenting cells (APCs). Once activated, they divide rapidly and secrete cytokines that regulate the immune response. T cells that express the CD8 protein on their surface are called T-cytotoxic cells (CTLs) and can destroy virally infected cells and tumor cells with toxic granules, and are also implicated in transplant rejection. Memory T cells are a subset of antigen-specific T cells that persist long-term after an infection has resolved. They quickly expand to large numbers of effector T cells upon re-exposure to their cognate antigen, thus providing the immune system with "memory" against past infections. Regulatory T cells, formerly known as suppressor T cells, are crucial for the maintenance of immunological tolerance. Their major role is to regulate T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus.
$\gamma\delta$ T Cells	This small group of T cells possesses a distinct T cell receptor (TCR) on their surface. It seems that these cells are able to recognize whole proteins rather than requiring peptides to be presented by MHC molecules on APCs. $\gamma\delta$ T cells utilize a variety of different mechanisms to regulate the inflammatory response. They secrete cytokines and mediate inflammatory balance by inducing apoptosis in opposing cell populations (cytotoxicity).
NK Cells	NK cells play a major role in the rejection of tumors and cells infected by viruses. The cells kill by releasing small cytoplasmic granules of proteins called perforins and granzymes. They can also kill target cells by mean of antibody dependent cell mediated cytotoxicity (ADCC).
Dendritic Cells	Upon activation, dendritic cells phagocyte and process antigens, migrate to lymph nodes to present the antigens to antigen-specific T cells and secrete various cytokines like IL-12 [9] and INF-γ [10] and chemokines that initiate and/or enhance many T and B lymphocyte responses.

Recruitment of leukocytes from the circulation into inflammatory tissues or tumors requires a series of soluble and cell-bound interactions between the responding leukocyte and vascular endothelial barrier. Chemotactic factors are believed to be responsible for this selective adhesion and transmigration. A superfamily of small, soluble, structurally-related molecules called ‘chemokines’ has been identified and shown to selectively promote the rapid adhesion and chemotaxis of a variety of leukocyte subtypes both *in vivo* and *in vitro*. In addition, bacterial wall-derived peptides, lipids, complement fragments as well as micro-milleu components such as ATP, ADP, adenosine, spingosine-1-phosphate, lysophosphatidic acid, histamine and serotonin have chemotactic activity for certain subtypes.

Most chemotaxins in leukocytes develop their biological activity by interactions with specific G_i protein-coupled serpentine receptors (GPCRs). The coupled guanine nucleotide-binding proteins (G proteins) represent a family of heterotrimeric proteins composed of α -, β -, and γ subunits. In humans, there are at least 21 $G\alpha$ subunits, 6 $G\beta$ subunits and 12 $G\gamma$ subunits [11]. The α -subunits are further subdivided in α_i , α_s , α_q and α_{12} subgroups. Recently, a fifth $G\alpha$ protein, G_v , was discovered [12]. Heterotrimeric G proteins function as "molecular switches," alternating between an inactive guanosine diphosphate (GDP)- and active guanosine triphosphate (GTP)-bound state in order to

regulate downstream signaling pathways and cell processes. They are activated in response to a conformation change in the G protein-coupled receptor after interactions with the specific ligands, followed by the exchange of GDP for GTP on the α -subunits. This results in the dissociation into active GTP-bound α -subunits and free $\beta\gamma$ -dimers. These components again activate other proteins in signal transduction pathways. The $G\alpha_i$ subgroup is named in accordance to their inhibitory function on adenylylcyclase. Free $\beta\gamma$ -subunits activate phospholipase $C\beta_2$ [13]. This enzyme cleaves phosphatidylinositol (4,5)-bisphosphate (PIP₂) to form two second-messenger molecules called inositol triphosphate (IP₃) and diacylglycerol (DAG). The latter activates another enzyme called protein kinase C (PKC), and IP₃ triggers the release of calcium from intracellular stores. Free $\beta\gamma$ -subunits are also able to activate phosphatidylinositol-3-kinase- γ , which phosphorylates PIP₂ generating PIP₃. This lipid is able to interact with pleckstrin homology domains (PH-domains) on a broad spectrum of effector proteins such as proteins of the serine/threonine-specific protein kinase family AKT. Once correctly positioned in the membrane *via* binding of PIP₃, Akt is phosphorylated on two key residues: T308 in the activation loop by PDK1 [14] and S473 in the hydrophobic motif of the C-terminal. The mammalian target of rapamycin complex 2 (mTORC2) acts as the long-sought PDK2 molecule [15]. Phosphorylation by mTORC2 stimulates the subsequent phosphorylation of Akt by PDK1. Activated Akt can then go on to activate or deactivate its myriad substrates *via* its kinase activity. Besides being a downstream effector of PI 3-kinases, Akt may also be activated in a PI 3-kinase-independent manner. Studies have suggested that cAMP-elevating agents could activate Akt through protein kinase A (PKA), although these studies are disputed and the mechanism of action is unclear. Akt regulates different cellular responses (e.g., cell proliferation, cell survival, and metabolism) by binding and regulating several downstream effectors. Akt can influence cell survival by regulating proapoptotic proteins like the BCL-2 family member BAD [16] or the indirect activation of nuclear factor κ B (NF- κ B) by regulating I κ B kinase (IKK). Akt-mediated phosphorylation of p53-specific E3 ubiquitin ligase (MDM2) results in degradation of p53. The inhibitory phosphorylation of glycogen synthase kinase 3 (GSK-3) by Akt leads to promotion of glycogen synthesis.

Activation of GPCR is also closely associated with the activation Rho family GTPases. Rho/Rac proteins constitute a subgroup of the Ras superfamily of GTP hydrolases. These proteins are classified into six subfamilies: Rho, Rac, Cdc42, Rnd, RhoBTB and RhoT/Miro. The “molecular switches” between the GDP- or GTP-bound state is controlled by two types of regulatory proteins GEFs and GAPs. GEFs induce the exchange of GDP for GTP molecules, promoting the activation of these proteins during signal transduction. The hydrolysis of the bound GTP molecules by GAPs, results in the transfer of the GTPase back to the inactive state. GEFs like P-Rex1 or SWAP70 can be activated by PIP₃ since they contain the PH domain. The Rho/Rac-GTPases coordinate diverse cellular functions including cytoskeletal events, cell polarity, vesicular trafficking, cell cycle and transcriptomal dynamics. In leukocytes, Rho proteins are essential for epithelial barrier functions, immune cell migration, adhesion, phagocytosis, superoxide production, cytokine secretion and immune cell signaling. This functional diversity can be explained by the interaction of Rac/Rho proteins with many downstream effector molecules. To date, more than 70 potential effectors have been identified e.g., PAK1, Stat3, FlnA (reviewed in [17]).

2. Pertussis Toxin

Pertussis toxin (PT) is a protein-based exotoxin produced by the bacterium *Bordetella pertussis*. It is involved in the colonization of bacteria in the respiratory tract and causes whooping cough. The exotoxin contains six subunits (named S1 through S5 - each complex contains two copies of S4). The subunits are arranged in an A-B structure: the A component is enzymatically active and is formed from the S1 subunit, while the B component is the receptor-binding portion and is made up of subunits S2–S5 [18].

PT is released from *Bordetella pertussis* in an inactive form. After binding to a cell membrane receptor, it is taken up in an endosome and retrograde transported to the trans-Golgi network and endoplasmic reticulum [19]. At some point during this transport, the A subunit becomes activated, presumably involving the action of glutathione and ATP. PT catalyzes the ADP-ribosylation of $G\alpha_i$ and $G\alpha_o$ subunits. This prevents the G proteins from interacting with G protein-coupled receptors on the cell membrane, interrupting intracellular communication. Since the $G\alpha$ subunits remain in their GDP-bound and inactive state, they cannot inhibit adenylyl cyclase, thereby causing adenylyl cyclase to be inappropriately active and leading to increased cellular concentrations of cAMP within the cell. On the other hand, serpentine receptors interact probably in a very specific manner with a distinct composition of α -, β -, and γ -subunits. Therefore, participation of $G\alpha_i$ in specific receptor-mediated signaling can be also seen in $\beta\gamma$ -regulated pathways (e.g., PLC β or PI3K γ) due to missing dissociation of the inactive heterotrimeric GDP-bound complex into free $\beta\gamma$ -dimers. Using pertussis toxin as a tool, essential participation of $G\alpha_i$ in multiple cell responses and signaling pathways has been reported in leukocytes (Table 2).

Table 2. Effect of pertussis toxin on different leukocyte functions. + is an increase or induction, - is an inhibition or decrease of different cell functions.

Cell Type	Cell functions	Pertussis toxin
Monocytes	Phagocytosis	+/- [27,28,44]
	Cytokine production	+/- [20,23,31]
	Chemotaxis	- [21,22,24,30,33]
	Migration	+ [29,34]
Neutrophils	Cytotoxicity	- [25]
	Phagocytosis	-[44,45]
	Oxygen reactive species	- [39,40–42,45]
	Degranulation	- [36]
	Migration	- [37,43]
	Chemotaxis	- [35,38,39,45]
B Cells	Proliferation	+/- [49,51,55]
	Activation	- [50,53,54,56,57]
	Antibody production	- [51]
	Migration	- [46–48,52]

Table 2. Cont.

$\alpha\beta$ T Cells	Proliferation	+ [32,60]
	Cytokine production	+/- [60,65,71,72]
	Migration	- [61,64,69,70]
	Chemotaxis	- [58,62,67,69]
	Th ₁ selection	+ [66]
	Th ₂ differentiation	+ [59]
$\gamma\delta$ T Cells	Proliferation	+ [68]
NK Cells	Cytotoxicity	- [81]
	Chemotaxis	- [73–80,82–84]
Dendritic Cells	Antigen presentation	- [89]
	Cytokine production	- [26,88]
	Migration	- [85,86,89]
	Chemotaxis	- [63,87,90]

3. Cholera Toxin

Cholera toxin is an oligomeric protein complex secreted by *Vibrio cholerae*. It consists of six protein subunits: a single copy of the A subunit (part A), and five copies of the B subunit (part B). The two parts are connected by a disulfide bond. The five B subunits form a five-member ring. The A subunit has two important segments. The A1 portion of the chain is an ADP-ribosyltransferase for Gs proteins, while the alpha helix form of the A2 chain sits in the central pore of the B subunit ring [91].

Cholera toxin bound to GM1 gangliosides on the surface of cell is endocytosed, and transported to the Golgi and the endoplasmic reticulum as a holotoxin. At the endoplasmic reticulum, a protein disulfide isomerase unfolds and cut the A1 fragment from the B subunits. Then, at least the A1 subunit is translocated into the cytosol via receptor-mediated endocytosis [92]. The free A subunit binds with a partner protein called ADP-ribosylation factor 6, driving a conformation change. Thereafter, the A-subunit catalyses ADP ribosylation from NAD to the G α s subunit of heterotrimeric G protein, resulting in constitutive cAMP production of the regulatory component of adenylate cyclase. Increased adenylate cyclase activity results in elevated intracellular cyclic AMP (cAMP) production and levels. The pathophysiological consequence of intestinal infection or elevated cAMP levels is therefore secretion of H₂O, Na⁺, K⁺, Cl⁻, and HCO₃⁻ into the lumen of the small intestine, resulting in rapid dehydration and diarrhea. Using cholera toxin as a tool, regulatory functions of G α s proteins and adenylyl cyclase in multiple cell responses has been reported in leukocytes (Table 3).

Table 3. Effect of cholera toxin on different leukocyte functions. + is an increase or induction, - is an inhibition or decrease, ne means no effect, and the other are simply several Cholera toxin effects on different cell functions.

Cell Type	Cell functions	Cholera toxin
Monocytes	Phagocytosis	-/ne [44,112,113]
	Cytokine production	+/- [93,94,96–103,109–111]
	Oxygen reactive species	ne [108]
	Chemotaxis	ne [105–107]
	Migration	ne [104]

Table 3. Cont.

Neutrophils	Phagocytosis Oxygen reactive species Degranulation Migration Chemotaxis	-/ne [112,119,124,125] -/ne [116,117,120,121,123] ne [122,123,126] -/ne [114,115,126] - [118,127–129]
B Cells	Proliferation Immunoglobulin production Memory cells	+/- [131,135,148–150,173,179] + [130,131,134–136,138,142–144,147,151,152,155,171] + [133,141,146,153]
$\alpha\beta$ T Cells	Proliferation Activation Cytotoxicity Cytokine production Chemotaxis Th ₁ selection Th ₂ differentiation Tolerance or memory	+/- [131,136,157,162,164,165,167,169,172,175,177–179,197] +/- [140,143,160,171–174,176] + [174] +/- [72,78,96,101,154,156,160–164,172] - [183] - [95,101,159,161,165,166] + [95,101,144,145,156,158,168,170] + [132,146]
NK Cells	Proliferation Activation Cytotoxicity Migration Chemotaxis	- [139] - [181,185] - [81,182,184,186,187] - [180] - [83,84,181]
Dendritic Cells	Activation Antigen presentation Cytokine production Migration	+ [194–196,200] + [191,197] +/- [93,101,161,189,190,192,194,196,199] + [137,188,193,199]

4. Toxin A and B

Toxin A and Toxin B (TcdA and TcdB) are the major virulence factors of *Clostridium difficile*. TcdA and Tcd B are exotoxins and belong to the family of clostridial glucosylation toxins. They are very large proteins with a molecular weight between 269 and 308 kDa and are characterized by a modular, tripartite composition [201]. The glucosyltransferase activity of the toxins is located in the N-terminal region. The hydrophobic region in the center of the protein is thought to be involved in the translocation of the toxin from the endosomes into the cytosol of the target cells. The receptor binding seems to be restricted to the C-terminal part.

Clostridial glucosylating toxins enter eukaryotic target cells according to the ‘short trip model’ of bacterial exotoxin uptake [202]. After binding to the target cell, they are endocytosed. In the endosomal compartment, the toxin undergoes conformational changes allowing the insertion in the endosomal membrane and subsequent pore formation in a pH-dependent manner. After autocatalytical cleavage only the glucosyltransferase domain of the N-terminus is released into the cytosol (reviewed by [203]).

The intracellular target of TcdA and TcdB are small GTPases of the Rho family. The toxins modify Rho GTPases via mono-O-glycosylation at a threonine residue, which is located in the switch-I region [204]. TcdA and TcdB specifically glucosylate RAC, Cdc42 or Ras [203]. The functional consequence

of the glucosylation is diverse, but it always causes the biological inactivity of the GTPases. The glucosylation blocks the activation of the GTPases by their activators (GEFs) and inhibits intrinsic and GAP-stimulated GTPase activity. The glucosylated Rho GTPase is not able to interact with GDI and is therefore located at the plasma membrane. The consequence of the glucosylation is the inhibition of the interaction with the effector proteins (kinases or adaptor proteins) with subsequent blocking of signal transduction pathways (Figure 2). In contrast to endogenous cytosolic mono-O-glucosylation, the glucosylation by TcdA and TcdB seems to be irreversible.

Inactivation of the Rho proteins leads to drastic changes in eukaryotic cells. The actin cytoskeleton is largely redistributed, accompanied by shrinking, rounding and detachment of the target cells. Using TcdA and TcdB as a tool, several more cellular responses have been reported (Table 4).

Figure 2. The glucosylation blocks (a) the activation of the GTPases by their activators (GEFs) and (b) inhibits intrinsic and GAP-stimulated GTPase activity. (c) The glucosylated Rho GTPase is not able to interact with GDI and is therefore located at the plasma membrane. (d) The consequence of the glucosylation is the inhibition of the interaction with the effector proteins (kinases or adapter proteins) with subsequent blocking of signal transduction pathways.

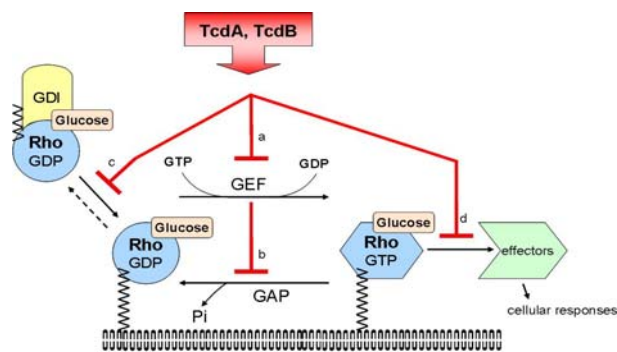


Table 4. Effect of *Clostridium difficile* Toxin A and Toxin B (TcdA and TcdB) on different leukocyte functions. + is an increase or induction, - is an inhibition or decrease, ne means no effect, and the other are several TcdA and TcdB reported effects on different cell functions.

Cell Type	Cell functions	<i>Clostridium difficile</i> toxins A and B
Monocytes	Phagocytosis	ne [205,214]
	Cytokine production	+ [206,208–210,212–215]
	Migration	- [207,211]
Neutrophils	Phagocytosis	-/ne [216,222]
	Oxygen reactive species	- [218,219]
	Migration	+ [209,212,213,219]
	Chemotaxis	- [220]
$\alpha\beta$ T Cells	Proliferation	- [221]
NK Cells	Cytotoxicity	+ [226]
Dendritic Cells	Maturation	+ [217]
	Phagocytosis	- [224,225]
	Migration	- [223]

5. Conclusions

Based on the literature using the toxins described here, Gi proteins are involved in processes like cytokine production, chemotaxis, degranulation and production of oxygen reactive species in leukocytes; also in the proliferation and antibody production of lymphocytes; and in the development of Th₁/Th₂ immunobalance. On the other hand, Gs proteins limit the cytotoxicity of NK cells, play a major role in Th₁/Th₂ balance and development of memory B cells. Finally, the glucosylation and blockade of the small GTPases of the Rho family affects several functions like chemotaxis and oxygen reactive species production of leukocytes, and the proliferation of lymphocytes.

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