



MB Fluid

Bacterial substances and cytokines

MBVax Bioscience Inc.
www.mbvax.com

Bacterial Substances

MB Fluid consists of a mixture of two types of killed bacteria: *Streptococcus pyogenes* is Gram-positive (meaning a triphenylmethane dye called Gram stain is not decolorized by ethanol) and *Serratia marcescens* is Gram-negative (the Gram stain is decolorized).

Gram-positive and Gram-negative bacteria contain different sets of substances that stimulate the innate immune system:

- Gram-negative bacteria contain unmethylated CpG DNA sequences, the endotoxin lipopolysaccharide (LPS), and peptidoglycan (PGN).
- Gram-positive bacteria also contain unmethylated CpG DNA sequences and peptidoglycan (PGN), but do not contain LPS.
- Gram-positive bacteria also contain exotoxins, lipoteichoic acid (LTA), streptolysin O (SLO) and cytoplasmic membrane-associated protein (CAP).
- In addition, both types of bacteria contain other, unidentified immune stimulatory substances.

Bacterial DNA

Bacterial DNA contains unmethylated CpG sequences that bind to the human Toll-like receptor TLR9 (Bauer¹) and trigger an innate immune response leading to the secretion of IL-6, IL-10, IL-12, IP-10, TNF- α , IFN- α , IFN- β and IFN- γ .² Both CD4-positive and CD4-negative peripheral blood dendritic precursor cells respond to CpG DNA, but monocyte-derived DCs did not respond to CpG (Hartmann³).

Endotoxin (LPS)

LPS activates cells through the pattern recognition receptors CD14 and Toll-like receptor 2 (TLR2) on monocytes, macrophages, endothelium and polymorphonuclear neutrophils, thereby inducing the release of TNF- α , IL-6, and nitric oxide (Dziarski⁴, Matsuura⁵). Nitric oxide is cytostatic and/or cytolytic for tumor cells (Farias-Eisner⁶). LPS also induces the production of IL-1 α , IL-8, IL-10 (Bjork⁷), IP-10 (Luster⁸), and small quantities of TNF- β (Hackett⁹), and activates the complement pathway (Loos¹⁰). LPS is a B cell mitogen and polyclonal activator in mice (Dziarski¹¹). Monocyte-derived DCs are highly sensitive to LPS, but both CD4-positive and CD4-negative peripheral blood dendritic precursor cells show little response to LPS (Hartmann¹²).

Exotoxins (Spe)

Streptococcal pyrogenic exotoxins (Spe) are produced in the cell walls of *Streptococcus pyogenes* and secreted into the extracellular environment. Exotoxins include SpeA, SpeB and SpeC, and a number of other exotoxins including SpeF, SpeG, SpeZ, SSA, SMEZ and SMEZ-2 (Muller-Alouf¹³). Exotoxins are both pyrogenic (induces a fever) and mitogenic (induces cellular proliferation). Exotoxins are pyrogenic because they stimulate the production of cytokines and chemokines. Exotoxins are mitogenic because they function as “superantigens” which can give rise to polyclonal activation (Marrack¹⁴, Leonard¹⁵).

Superantigens have the ability to bind to major histocompatibility complex molecules on antigen-presenting cells and simultaneously to T cell receptors, thereby triggering a polyclonal expansion of T lymphocytes. The superantigen-mediated T cell activation process has also been shown to elicit a characteristic pattern of cytokines distinct from that seen with LPS, including the T cell derived cytokines, IFN- γ and IL-2 (Bjork¹⁶). TNF- β is induced more efficiently by the superantigens than by LPS (Hackett¹⁷).

The best-characterized exotoxin, streptococcal pyrogenic exotoxin type A (SpeA), stimulates the production of:

- Cytokines IL-1 α , IL-6, TNF- α , IL-12, IL-10, IP-10;
- Th1 derived cytokines TNF- β , IFN- γ , IL-2;
- Th2 derived cytokine IL-5;
- IL-3, GM-CSF;
- Chemokines IL-8, RANTES and MIP-1 α (Muller-Alouf¹⁸); and
- Enhances the host antibody response to other antigens (Hanna¹⁹).

Peptidoglycan (PGN)

Peptidoglycan, a major component of the cell walls of Gram-positive bacteria, induces the release of TNF- α (Dziarski²⁰) IL-8 (Wang²¹), IL-1 and IL-6 (Schwandner²²). PGN is a B cell mitogen and polyclonal activator in mice (Dziarski²³). PGN is also a constituent of the cell walls of Gram-negative bacteria.

Lipoteichoic acid (LTA)

Lipoteichoic acid also binds to CD14 (Dziarski, 1998), inducing release of TNF. LTA induces TNF- α , IFN- α , IFN- β and IFN- γ in primed mice (Tsutsui²⁴); IL-1 β , IL-6 and TNF in human monocyte cultures (Bhakdi²⁵, Keller²⁶, Yamamoto²⁷); IL-8 and MIP-1 α (Gao²⁸); and IL-12 (Cleveland²⁹). LTA stimulates mitogenesis of T, but not B, lymphocytes (Beachey³⁰), and activates the complement pathway (Loos³¹).

Streptolysin O (SLO)

Streptolysin O, produced by *Streptococcus pyogenes*, stimulates monocytes to produce IL-1 β and TNF- α (Hackett³²), and stimulates bone marrow-derived mast cells to produce IL-4, IL-6, IL-13, GM-CSF, TNF- α and MCP-1 (Stassen³³), and binds IgG antibodies to form immune complexes with potent complement-activating capacity (Bhakdi³⁴).

Cytoplasmic membrane-associated protein (CAP)

CAP is found in the cytoplasmic membrane but not in cell walls, peptidoglycan, lipoteichoic acids, or cytoplasmic soluble fractions. This mitogenic factor produces polyclonal activation of many classes of T lymphocytes (Itoh³⁵).

Histone-like protein (HlpA)

HlpA is a constituent of *Streptococcus pyogenes*. Exposure of macrophages to a mixture of HlpA and lipoteichoic acid resulted in a synergistic response in the production of both TNF- α and IL-1 (Zhang³⁶).

Dependent effects

The immune responses to bacterial substances are complex.

- **Bacterial substances can synergistically enhance immune responses.**
 - TNF alone has a low systemic toxicity in tumor- and pathogen-free mice. However, TNF given intravenously with nanogram quantities of LPS can cause lethal shock (Rothstein³⁷). Additional synergy might be expected to occur from the presence of LPS and the streptococcal exotoxin itself (Kim³⁸), a similar combination of which has been recommended and exploited as a method for the detection of LPS, the lethality of which was found to be enhanced by as much as 50,000-fold or more (Bohach³⁹).
 - Synergistic induction of TNF and IL-1 from macrophages has been observed *in vitro* under combined treatment with LPS and superantigens (Parsonnet⁴⁰, Beezhold⁴¹). In terms of IL-1 β production from human monocytes, SpeA and Streptolysin O together were synergistic: SpeA 193 pg/ml; Streptolysin O, 452 pg/ml; SpeA plus Streptolysin O, 799 pg/ml (Hackett⁴²).
 - Streptococcal pyrogenic exotoxins can enhance the host antibody response to other antigens (Hanna⁴³).

- **Bacterial substances can antagonistically reduce immune responses.**
 - Peptidoglycan-induced monokine production can be blocked by LPS (Weidemann⁴⁴).

- **Bacterial substances can differentially induce proliferation of lymphocytes.**
 - Exotoxins include the classical Streptococcal pyrogenic exotoxins type A, B and C, and a number of other exotoxins including type F, type G, type Z, SSA, SMEZ and SMEZ-2. These exotoxins bind to different T cell receptor motifs and thereby stimulate the expansion of different polyclonal populations of T cells (Muller-Alouf⁴⁵).
 - Lipoteichoic acid is also mitogenic for T cells. Both T and B lymphocytes possess a single population of specific binding sites of lipoteichoic acid, and as a consequence of its binding, lipoteichoic acid stimulates mitogenesis of T, but not B, lymphocytes (Beachey⁴⁶).
 - Cytoplasmic membrane-associated protein (CAP) also produces polyclonal activation of many classes of T lymphocytes (Itoh⁴⁷).

- **Bacterial substances can differentially induce the maturation of antigen-presenting dendritic cells (DCs).**
 - Both CD4-positive and CD4-negative peripheral blood dendritic precursor cells respond to CpG DNA, but these DCs showed little response to LPS. In contrast, monocyte-derived DCs did not respond to CpG, but they were highly sensitive to LPS (Hartmann⁴⁸).

- **Bacterial substances induce the production of cytokines via different pathways.**
 - The LPS receptor – CD14 – also binds lipoteichoic acid, inducing release of TNF (Dziarski⁴⁹); but peptidoglycan (which also induces TNF) interacts via a different receptor because blocking CD14 had no influence on Peptidoglycan induced TNF (Wang⁵⁰). In mice, lipoteichoic acid suppressed Meth A fibrosarcoma tumor growth and Peptidoglycan did not – also lipoteichoic acid induced TNF in *Propionibacterium acnes*-primed mice, but Peptidoglycan did not (Usami⁵¹).

- **Bacterial substances induce the production of cytokines with different kinetics.**
 - The kinetics of TNF- α production after stimulation is different for LPS, Streptolysin O and SpeA. LPS immediately stimulates production, rising to a max in 24 h then leveling off through 72 h. Production due to SpeA and Streptolysin O does not begin for 6 h, then rises following similar

patterns until 48 h, then SpeA continues to rise while Streptolysin O falls and at 72 h is comparable to its level at 12 h (Hackett,⁵² Fast⁵³).

Cytokines

The biological activity of MB Fluid can be described in terms of the cytokines and other substances that mediate the immune response.

GM-CSF

Granulocyte-macrophage colony-stimulating-factor is a cytokine that stimulates proliferation of granulocytes and macrophages, activates macrophages and promotes the differentiation and maturation of dendritic cells. Activated T cells, macrophages, endothelial cells and bone marrow stromal cells produce GM-CSF.

Oncolytic properties of GM-CSF

In the treatment of cancer, GM-CSF produced a 50% reduction in tumor volume in a soft tissue sarcoma patient (Steward⁵⁴). Injection of a murine tumorigenic T-leukemia cell line expressing mGM-CSF into pre-established tumors of syngenic mice led to a significant regression of these tumors (Hsieh⁵⁵). Furthermore, syngenic mice injected with melanoma cells or cells transfected with a recombinant GM-CSF gene either completely rejected the tumor cells or developed tumors with a mean volume fifty-times smaller than the control (Armstrong⁵⁶).

IL-1 α , IL-1 β

There are two forms of the cytokine interleukin-1, IL-1 α and IL-1 β , coded by separate genes and showing only 30% structural homology. Nevertheless, these two cytokines bind the same receptors and have the same function: to induce and promote inflammatory reactions. IL-1 is produced by activated macrophages, and in smaller quantities by neutrophils, epithelial cells (especially keratinocytes), and endothelial cells.

Oncolytic properties of IL-1

When human IL-1 β was introduced into B16 mouse melanoma cells, the growth of B16 transfectants injected subcutaneously in syngenic mice was significantly reduced (Bjorkdahl⁵⁷).

IL-2

The cytokine IL-2 is the major growth factor for antigen-activated T lymphocytes; it also promotes B lymphocyte proliferation, antibody production, and activates NK cells. IL-2 is produced by activated T lymphocytes, mostly CD4⁺ T cells and in smaller quantities by CD8⁺ T cells.

Oncolytic properties of IL-2

The FDA approved high-dose IL-2 for treatment of patients with metastatic kidney cancer in 1992 and for metastatic melanoma in 1998 (Rosenberg⁵⁸).

IL-3

IL-3 acts on immature bone marrow progenitors to stimulate the production of lymphocytes. IL-3 is produced by CD4⁺ T lymphocytes.

IL-4

IL-4 participates in the activation of B-cells as well as other cell types. It is a co-stimulator of DNA-synthesis, induces the expression of class II MHC molecules on resting B-cells, and also enhances both secretion and cell surface expression of IgE and IgG1. IL-4 also stimulates cytotoxic *T lymphocytes* (CTLs). IL-4 is secreted by T_h1 cells (T helper cells, type 1).

Oncolytic properties of IL-4

IL-4 augments tumor immunogenicity and enhances the induction of tumor reactive lymphoid cells in animal models (Krauss⁵⁹). Gene transfer of IL-4 into mouse tumor cells has been shown to stimulate a strong immune response resulting in the rejection of the transduced tumor when injected *in vivo* (Melani⁶⁰). Phase I/II clinical trials have been conducted in which human autologous dermal fibroblasts were cultured, transduced with the IL-4 gene, selected, irradiated, and administered to patients as a vaccine (Elder⁶¹).

IL-5

The cytokine IL-5 stimulates the growth and differentiation of eosinophils, activates mature eosinophils, and stimulates the production of B lymphocytes and IgA antibodies. The principal sources of IL-5 are the T_h2 subset of activated CD4⁺ T lymphocytes and activated mast cells.

IL-6

IL-6 is a cytokine that plays a major role in inflammation, stimulates the synthesis of acute phase proteins by hepatocytes, and serves as a growth factor for cells of the B-cell lineage, especially terminally differentiated Ig-secreting plasma cells. Mononuclear phagocytes, endothelial cells, fibroblasts, and other cells, in response to bacterial substances and to other cytokines notably IL-1 and TNF, produce IL-6.

Oncolytic properties of IL-6

In SCID mice bearing human tumors and reconstituted with human CTL, administration of a recombinant adenoviral vector expressing IL-6 induced human CTL and inhibited growth and metastasis of the human tumor cells (Saggio⁶²).

IL-8

IL-8 is a chemokine that attracts neutrophils, basophils, and *T cells*, but not monocytes. It is also involved in neutrophil activation and is released from several cell types in response to an inflammatory stimulus. Leukocytes and several types of tissue cells produce IL-8.

Oncolytic properties of IL-8

Human IL-8 dramatically inhibited the tumor growth rate of CHO cells *in vivo* when injected into nude mice (Hirose⁶³).

IL-10

The cytokine IL-10 has potent anti-inflammatory properties. IL-10 is the major inhibitor of activated macrophages. IL-10 inhibits the production of macrophage-derived IFN- γ , IL-2, IL-3, TNF and GM-CSF, thereby suppressing inflammation and the T_h1 pathway of T helper cell differentiation, and serving as negative feedback in macrophage activation. IL-10 plays a role in adaptive immunity by enhancing the proliferation of B lymphocytes. IL-10 is produced by activated macrophages.

Oncolytic properties of IL-10

Gene transfer studies have suggested that IL-10 induced tumor suppression is mediated via enhanced natural killer (NK) cell activity (Gerard⁶⁴, Kundu⁶⁵) as well as inducible isoforms of nitric oxide synthase (Kundu⁶⁶).

IL-12

The cytokine IL-12 is the principal mediator of early innate immune responses to bacterial substances. The biological role of IL-12 is to initiate a series of responses involving macrophages, NK cells, and T lymphocytes. It is a potent stimulator of the T_H1 pathway of helper T cell differentiation, stimulates production of IFN- γ by NK cells and T lymphocytes, and enhances the cytolytic functions of activated NK cells and $CD8^+$ cytotoxic T lymphocytes (CTLs). The two principal sources of IL-12 are activated macrophages and dendritic cells.

Oncolytic properties of IL-12

The antitumor activity of IL-12 is documented by a large set of data from numerous mouse models (Cavallo⁶⁷). Gene transfer studies of IL-12 have been efficient at reducing tumor growth and even complete eradication of established primary tumors, as well as reduction of metastases in different tumor models (Hiscox⁶⁸). Also, IL-12 expression at the tumor site generated a long-term protective antitumor immune response. IL-12 gene transfer is being tested in human clinical trials (Sun⁶⁹).

IL-13

The cytokine IL-13 suppresses macrophage activation and antagonizes IFN- γ . IL-13 also induces the differentiation of dendritic cells. T_H2 cells and some epithelial cells produce IL-13.

Oncolytic properties of IL-13

IL-13 gene transfer induces anti-tumor protection due to the stimulation of specific antitumor effector cells (Lebel-Biany⁷⁰).

Interferon

IFN- α and IFN- β , despite their structural differences, bind the same type I interferon receptor and are therefore called type I interferon. IFN- α , sometimes called leukocyte interferon, comprises a family of 20 species of molecules that are produced by a subset of mononuclear phagocytes. IFN- β , a single substance produced by a variety of cell types, most notably fibroblasts, is also called fibroblast interferon.

IFN- γ , a single substance, is also called immune interferon or type II interferon. It exerts numerous biological effects including activating macrophages, enhancing the expression of class I and class II MHC molecules, promoting the differentiation of naïve $CD4^+$ T cells to the T_H1 subset, inhibiting the proliferation of T_H2 cells, promoting the antibody class switch to IgG subclasses, inhibiting the class switch to

IgE, activating neutrophils, and enhancing the cytolytic activity of NK cells. NK cells, CD4⁺ T_h1 cells and CD8⁺ cells produce IFN- γ .

Oncolytic properties interferon

IFN- α is an FDA approved treatment for hairy cell leukemia and melanoma, and is being used as an investigational drug for numerous other cancers.

IFN- γ induces macrophages to release NO, which is cytostatic and/or cytolytic for tumor cells (Farias-Eisner⁷¹).

All types of interferon enhance the expression of MHC class I antigens and promote the T_h1 pathway of T helper differentiation by target cells, and induce target cells to display the same class of immune epitopes as displayed by antigen presenting cells such as dendritic cells, thereby allowing the detection and destruction of tumor cells that might have otherwise been invisible to the immune system (Van den Eynde⁷²).

IP-10

Interferon-inducible protein-10 is a member of the chemokine family. IP-10 exerts a chemotactic activity on lymphoid cells such as T cells, monocytes and NK cells. IP-10 is also a potent inhibitor of angiogenesis: it inhibits neovascularization by suppressing endothelial cell differentiation. IP-10 is an IFN- γ inducible protein that is produced mainly by monocytes, but also by T cells, fibroblasts and endothelial cells.

Oncolytic properties of IP-10

Gene transfer of IP-10 into tumor cells reduced their tumorigenicity, and elicited a long-term protective immune response (Luster⁷³). The angiostatic activity of IP-10 was shown to mediate tumor regression: tumor cells expressing IP-10 became necrotic *in vivo* (Sgadari⁷⁴). IP-10 has also been shown to mediate the angiostatic effects of IL-12 that lead to tumor regression (Tannenbaum⁷⁵).

MCP-1

Monocyte chemoattractant protein-1 (MCP-1) is a chemokine produced by a variety of hematopoietic and non-hematopoietic cell types. MCP-1 attracts monocytes, T and NK cells.

Oncolytic properties of MCP-1

Gene transfer of MCP-1 into tumor cells demonstrated antitumor effects (Manome⁷⁶).

MIP-1 α

Macrophage inflammatory protein-1 α is a chemokine. MIP-1 α attracts monocytes, neutrophils, eosinophils, dendritic cells, NK, and T cells.

Oncolytic properties of MIP-1 α

MIP-1 α exerts an antitumoral effect because of its ability to recruit immune cells at the tumor site. In mice, MIP-1 α elicited a long-term immune response that resulted in protection of the animals against challenge by tumor cells (Nakashima⁷⁷).

RANTES

RANTES is a chemokine that attracts monocytes, dendritic, T and NK cells, eosinophils and basophils.

Oncolytic properties of RANTES

Tumor cells transduced with the RANTES gene had a reduced ability to form tumors *in vivo*, and elicited an anti-tumor immune response that protected animals from challenge with the parent tumor cells (Mule⁷⁸).

TNF- α , TNF- β

Tumor necrosis factor alpha is a cytokine that induces and promotes inflammatory reactions involving recruitment of neutrophils and monocytes to the site of infection, and activation of these cells. Additionally, TNF- α stimulates endothelial cells, and also macrophages, to secrete chemokines that further increases the migration of leukocytes from blood to tissue. TNF- α also stimulates the secretion of IL-1 by macrophages. TNF also enhances the antibody response. LPS-activated macrophages, antigen-activated T lymphocytes, NK cells and mast cells produce TNF- α . The target of TNF- α is any cell (all human cell types express TNF receptors).

Tumor necrosis factor beta is similar in biological effect and structure to TNF (but it is a different molecule). Also called Lymphotoxin (LT), TNF- β is produced by some antigen-activated T lymphocytes in smaller quantities than the TNF- α made by macrophages, therefore TNF- β does not exert systemic effects but acts like a local promoter of inflammation. TNF- β is induced more efficiently by the superantigens than by LPS (Hackett⁷⁹).

Oncolytic properties of TNF

As its name implies, TNF has the ability to destroy tumors. Researchers have achieved 90% complete response rates by employment of isolated limb perfusion to deliver high local concentrations of TNF to selected patients with melanoma and sarcoma (Lienard⁸⁰). TNF has been shown to facilitate the *in vivo* localization of radiolabelled monoclonal antibodies at the site of the tumor to which they were directed (Smyth⁸¹).

Bacterial Substances and Immune Mediators								
	<i>Serratia marcescens</i>		<i>Streptococcus pyogenes</i>					
	<u>CpG</u>	<u>LPS</u>	<u>CpG</u>	<u>Spe</u>	<u>PGN</u>	<u>LTA</u>	<u>SLO</u>	<u>CAP</u>
GM-CSF				X			X	
IL-1		X		X	X	X	X	
IL-2				X				
IL-3				X				
IL-4							X	
IL-5				X				
IL-6	X	X	X	X	X	X	X	
IL-8		X		X	X	X		
IL-10	X	X	X	X				
IL-12	X		X	X		X		
IL-13							X	
IFN- α	X		X			X		
IFN- β	X		X			X		
IFN- γ	X		X	X		X		
IP-10	X	X	X	X				
MCP-1							X	
MIP-1 α				X		X		
RANTES				X				
TNF- α	X	X	X	X	X	X	X	
TNF- β		X		X				
Inducer of mitogenesis, enhancement or maturation of:								
T lymphocytes				X		X		X
B lymphocytes		X			X			
Dendritic cells	X	X	X					
Complement		X				X	X	

References

- ¹ Bauer S, Kirschning CJ, Hacker H, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. PNAS 2001; 98(16):9237.
- ² “CpG mechanism of action” www.coleypharma.com/wt/coley/cpg_action Accessed Dec 17, 2003.
- ³ Hartmann G, Weiner GJ, Krieg AM. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. PNAS 1999; 96:9305.
- ⁴ Dziarski R, Tapping R, Tobias PS. Binding of bacterial peptidoglycan to CD14. J Biol Chem 1998; 273:8680.
- ⁵ Matsuura M, Kiso M, Hasegawa A. Activity of monosaccharide lipid A analogues in human monocytic cells as agonists or antagonists of bacterial lipopolysaccharide. Inf and Immunity 1999; 67(12):6286.
- ⁶ Farias-Eisner R, Sherman MP, Aeberhard E, Chaudhuri G. Nitric oxide is an important mediator for tumoricidal activity in vivo. PNAS 1994; 91:9407.
- ⁷ Bjork L, et al. Endotoxin and *Staphylococcus aureus* enterotoxin A induce different patterns of cytokines. Cytokine 1992; 4:513.
- ⁸ Luster, A.D., and Leder, P. (1996) IP-10, a CXC chemokine, elicits a potent thymus-dependent antitumor response in vivo. J. Exp. Med. 178: 1057-1065.
- ⁹ Hackett SP, Stevens DL. Superantigens associated with staphylococcal and streptococcal toxic shock syndrome are potent inducers of tumor necrosis factor-beta synthesis. J Infect Dis 1993; 168:232.
- ¹⁰ Loos M, Clas F, Fischer W. Interaction of purified lipoteichoic acid with the classical complement pathway. Inf Immunity 1986; 53(3):595.
- ¹¹ Dziarski R. Studies on the mechanism of peptidoglycan- and lipopolysaccharide-induced polyclonal activation. Inf Immunity 1982; 35(2):507.
- ¹² Hartmann G, Weiner GJ, Krieg AM. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. PNAS 1999; 96:9305.
- ¹³ Muller-Alouf H, Proft T, Zollner TM, et al. Pyrogenicity and cytokine-inducing properties of *Streptococcus pyogenes* superantigens: comparative study of streptococcal mitogenic exotoxin Z and pyrogenic exotoxin A. Inf and Immunity 2001; 69(6):4141.
- ¹⁴ Marrack P, Kappler J. The staphylococcal enterotoxins and their relatives. Science 1990; 248:705.
- ¹⁵ Leonard BA, et al. Cell and receptor requirements for streptococcal pyrogenic exotoxin *T cell* mitogenicity. Infect Immunity 1991; 59:1210.
- ¹⁶ Bjork L, et al. Endotoxin and *Staphylococcus aureus* enterotoxin A induce different patterns of cytokines. Cytokine 1992; 4:513.
- ¹⁷ Hackett SP, Stevens DL. Superantigens associated with staphylococcal and streptococcal toxic shock syndrome are potent inducers of tumor necrosis factor-beta synthesis. J Infect Dis 1993; 168:232.
- ¹⁸ Muller-Alouf H, Proft T, Zollner TM, et al. Pyrogenicity and cytokine-inducing properties of *Streptococcus pyogenes* superantigens: comparative study of streptococcal mitogenic exotoxin Z and pyrogenic exotoxin A. Inf and Immunity 2001; 69(6):4141.

- ¹⁹ Hanna EE, Watson DW. Enhanced immune response after immunosuppression by streptococcal pyrogenic exotoxin. *Infect Immun* 1973; 7:1009.
- ²⁰ Dziarski R, Tapping R, Tobias PS. Binding of bacterial peptidoglycan to CD14. *J Biol Chem* 1998; 273:8680.
- ²¹ Wang Q, Dziarski R, Kirschning CJ, et al. Micrococci and peptidoglycan activate TLR2>MyD88>IRAK>TRAF>NIK>IKK>NF-kappa-B signal transduction pathway that induces transcription of interleukin-8. *Inf Immunity* 2001; 69(4):2270.
- ²² Schwandner R, Dziarski R, Wesche H, et al. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem* 1999; 274(25):17406.
- ²³ Dziarski R. Studies on the mechanism of peptidoglycan- and lipopolysaccharide-induced polyclonal activation. *Inf Immunity* 1982; 35(2):507.
- ²⁴ Tsutsui O, Koikeguchi S, Matsumura T, Kato K. Relationship of the chemical structure and immunobiological activities of lipoteichoic acid from *Streptococcus faecalis* (*Enterococcus hirae*) ATCC 9790. *FEMS Microbiol Immunol* 1991; 76:211.
- ²⁵ Bhakdi S, Klonisch T, Nuber P, Fischer W. Stimulation of monokine production by lipoteichoic acids. *Infect Immun* 1991; 59:4614.
- ²⁶ Keller R, Fischer W, Keist R, Bassetti S. Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. *Infect Immunity* 1992; 60:3664.
- ²⁷ Yamamoto A, Usami H, Nagamuta M, et al. The use of lipoteichoic acid (LTA) from *Streptococcus pyogenes* to induce a serum factor causing tumor necrosis. *Br J Cancer* 1985; 51:739.
- ²⁸ Gao JJ, Xue Q, Zuvanich EG, et al. Commercial preparation of lipoteichoic acid contain endotoxin that contributes to activation of mouse macrophages in vitro. *Inf Immunity* 2001; 69(2):751
- ²⁹ Cleveland MG, Gorham JD, Murphy TL, et al. Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. *Inf Immunity* 1996; 64(6):1906.
- ³⁰ Beachey EH, et al. Lymphocyte binding and T cell mitogenic properties of group A streptococcal lipoteichoic acid. *J Immunol* 1979; 122:189.
- ³¹ Loos M, Clas F, Fischer W. Interaction of purified lipoteichoic acid with the classical complement pathway. *Inf Immunity* 1986; 53(3):595.
- ³² Hackett SP, Stevens DL. Streptococcal toxic shock syndrome: synthesis of tumor necrosis factor and interleukin-1 by monocytes stimulated with pyrogenic exotoxin A and streptolysin O. *J Infect Dis* 1992; 165:879.
- ³³ Stassen M, Muller C, Richter C, et al. The streptococcal exotoxin streptolysin O activates mast cells to produce tumor necrosis factor alpha by p38 mitogen-activated protein kinase- and protein kinase C-dependent pathways. *Inf Immunity* 2003; 71(11):6171.
- ³⁴ Bhakdi S, Tranum-Jensen J. Complement activations and attack on autologous cell membranes induced by streptolysin O. *Inf Immunity* 1985; 48(3):713.

- ³⁵ Itoh T, Satoh H, Isono N, et al. Mechanism of stimulation of T cells by *Streptococcus pyogenes*: isolation of a major mitogenic factor, cytoplasmic membrane-associated protein. *Inf and Immunity* 1992; 60(8):3128.
- ³⁶ Zhang L, Ignatowski TA, Spengler RN, et al. Streptococcal histone induces murine macrophages to product inteleukin-1 and tumor necrosis factor alpha. *Inf Immunity* 1999; 67(12):6473.
- ³⁷ Rothstein JL, Schreiber H. Synergy between tumor necrosis factor and bacterial products causes hemorrhagic necrosis and lethal shock in normal mice. *PNAS* 1988; 85:607.
- ³⁸ Kim YB, Watson DW. A purified group A streptococcal pyrogenic exotoxin – physicochemical and biological properties including the enhancement of susceptibility to endotoxin lethal shock. *J Exp Med* 1970; 131:611.
- ³⁹ Bohach GA, Schlievert PM. Detection of endotoxin by enhancement with toxic shock syndrome toxin-1 (TSST-1). *Meth Enzymol* 1988; 165:302.
- ⁴⁰ Parsonnet J, Gillis ZA. Production of tumor necrosis factor by human monocytes in response to toxic-shock-syndrome toxin-1. *J Infect Disease* 1988; 158:1026.
- ⁴¹ Beezhold DH et al. Synergistic induction of interleukin-1 by endotoxin and toxic shock syndrome toxin-1 using rat macrophages. *Infect Immunity* 1987; 55:2865.
- ⁴² Hackett SP, Stevens DL. Streptococcal toxic shock syndrome: synthesis of tumor necrosis factor and interleukin-1 by monocytes stimulated with pyrogenic exotoxin A and streptolysin O. *J Infect Dis* 1992; 165:879.
- ⁴³ Hanna EE, Watson DW. Enhanced immune response after immunosuppression by streptococcal pyrogenic exotoxin. *Infect Immun* 1973; 7:1009.
- ⁴⁴ Weidemann B, Brade H, Rietschel ET, et al. Soluble peptidoglycan-induced monokine production can be blocked by anti-CD14 monoclonal antibodies and by lipid A parital structures. *Inf Immunity* 1994; 62(11):4709.
- ⁴⁵ Muller-Alouf H, Proft T, Zollner TM, et al. Pyrogenicity and cytokine-inducing properties of *Streptococcus pyogenes* superantigens: comparative study of streptococcal mitogenic exotoxin Z and pyrogenic exotoxin A. *Inf and Immunity* 2001; 69(6):4141.
- ⁴⁶ Beachey EH, et al. Lymphocyte binding and T cell mitogenic properties of group A streptococcal lipoteichoic acid. *J Immunol* 1979; 122:189.
- ⁴⁷ Itoh T, Satoh H, Isono N, et al. Mechanism of stimulation of T cells by *Streptococcus pyogenes*: isolation of a major mitogenic factor, cytoplasmic membrane-associated protein. *Inf and Immunity* 1992; 60(8):3128.
- ⁴⁸ Hartmann G, Weiner GJ, Krieg AM. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *PNAS* 1999; 96:9305.
- ⁴⁹ Dziarski R, Tapping R, Tobias PS. Binding of bacterial peptidoglycan to CD14. *J Biol Chem* 1998; 273:8680.
- ⁵⁰ Wang JE, Jorgensen PF, Almlof M, et al. Peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* induce tumor necrosis factor alpha, inteleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model. *Inf and Immunity* 2000; 68(7):3965.

- ⁵¹ Usami H, et al. Antitumor effects of streptococcal lipoteichoic acids on Meth A fibrosarcoma. *Br J Cancer* 1988; 57:70.
- ⁵² Hackett SP, Stevens DL. Streptococcal toxic shock syndrome: synthesis of tumor necrosis factor and interleukin-1 by monocytes stimulated with pyrogenic exotoxin A and streptolysin O. *J Infect Dis* 1992; 165:879.
- ⁵³ Fast DJ, et al. Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. *Infect Immunity* 1989; 57:291.
- ⁵⁴ Steward WP, et al. Recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) given as daily short infusions – a phase I dose-toxicity study. *Br J Cancer* 1989; 59:142.
- ⁵⁵ Hsieh CL, Pang VF, Chen DS, Hwang LH (1997). Regression of established mouse leukemia by GM-CSF-transduced tumor vaccine: implications for cytotoxic T lymphocyte responses and tumor burdens. *Hum Gene Ther* 1997 Nov 1;8(16):1843-1854
- ⁵⁶ Armstrong, C. A., Botella, R., Galloway, T. H., Murray, N., Kramp, J. M., Song, I. S., and Ansel, J. C. (1996). Antitumor effects of granulocyte-macrophage colony-stimulating factor production by melanoma cells. *Cancer Res.* 56: 2191-2198.
- ⁵⁷ Bjorkdahl, O., Wingren, A. G., Hedhund, G., Ohlsson, L., and Dohlsten, M. (1997) Gene transfer of a hybrid interleukin-1 beta gene to B16 mouse melanoma recruits leucocyte subsets and reduces tumour growth in vivo. *Cancer Immunol. Immunother.* (1997) 44: 273-281
- ⁵⁸ Rosenberg SA. Progress in the development of immunotherapy for the treatment of patients with cancer. *J Intern Med* 2001; 250(6):462-75.
- ⁵⁹ Krauss, J. C., Cameron, M. J., Park, A. N., Forslund, K., and Chang, A. E. (1995). Efficient transduction of early passage human melanoma to secrete IL-4. *J. Immunol. Methods* 183: 239-250.
- ⁶⁰ Melani, C., Chiodoni, C., Arienti, F., Maccalli, C., Sule-Suso, J., Anichini, A., Colombo, M. P., and Parmiani, G. (1994). Cytokine gene transduction in tumor cells: interleukin (IL)-2 or IL-4 gene transfer in human melanoma cells. *Nat. Immun.* 13: 76-84.
- ⁶¹ Elder, E. M., Lotze, M. T., and Whiteside, T. L. (1996). Successful culture and selection of cytokine gene-modified human dermal fibroblasts for the biologic therapy of patients with cancer. *Hum. Gene. Ther.* 7: 479-487.
- ⁶² Saggio I, Ciapponi L, Savino R, et al. Adenovirus-mediated gene transfer of a human IL-6 antagonist. *Gene Ther.* 4: 839-845.
- ⁶³ Hirose K, Hakozaiki M, Nyunoya Y, et al. Chemokine gene transfection into tumour cells reduced tumorigenicity in nude mice in association with neutrophilic infiltration. *Br. J. Cancer* 1995; 72: 708-714.
- ⁶⁴ Gerard, CM, Bruyns C, Delvaux A, et al. Loss of tumorigenicity and increased immunogenicity induced by interleukin-10 gene transfer in B16 melanoma cells. *Hum. Gene. Ther* 1996; 7: 23-31.
- ⁶⁵ Kundu, N., and Fulton, A. M. (1997). Interleukin-10 inhibits tumor metastasis, downregulates MHC class I, and enhances NK lysis. *Cell. Immunol.* 180: 55-61.
- ⁶⁶ Kundu N, Dorsey R, Jackson MJ, Guitierrez P, Wilson K, Fu S, Ramanujam K, Thomas E, Fulton AM (1998) Interleukin-10 gene transfer inhibits murine mammary tumors and elevates nitric oxide. *Int J Cancer* 76(5):713-9

-
- ⁶⁷ Cavallo F, Di Carlo E, Butera M, et al. Immune events associated with the cure of established tumors and spontaneous metastases by local and systemic IL-12. *Cancer Res* 1999; 59:414-21.
- ⁶⁸ Hiscox, S. and Jiang, W.G. (1997). Interleukin-12, an emerging anti-tumour cytokine. *In Vivo* 11: 125-132.
- ⁶⁹ Sun, Y., et al. (1998). Vaccination with IL-12 gene-modified autologous melanoma cells: preclinical results and first clinical phase I study. *Gene Ther.* 5: 481-490.
- ⁷⁰ Lebel-Biany et al. 1995. Experimental gene therapy of cancer using tumor cells engineered to secrete interleukin-13. *Eur. J. Immunol.* 25: 2340-2348.
- ⁷¹ Farias-Eisner R, Sherman MP, Aeberhard E, Chaudhuri G. Nitric oxide is an important mediator for tumoricidal activity in vivo. *PNAS* 1994; 91:9407.
- ⁷² Van den Eynde BJ, Morel S. Differential processing of class-I-restricted epitopes by the standard proteasome and the immunoproteasome. *Current Opinion in Immunol* 2001; 13:147.
- ⁷³ Luster, A.D., and Leder, P. (1996) IP-10, a CXC chemokine, elicits a potent thymus-dependent antitumor response in vivo. *J. Exp. Med.* 178: 1057-1065
- ⁷⁴ Sgadari, S., et al. (1996) Interferon-inducible protein-10 identified as a mediator of tumor necrosis in vivo. *Proc. Natl. Acad. Sci. USA* 93: 13791-13796
- ⁷⁵ Tannenbaum, C.S., Raymond, T., Armstrong, D., Finke, J.H., Bukowski, R.M., and Hamilton, T.A. (1998) The CXC chemokines IP-10 and Mig are necessary for IL-12-mediated regression of the mouse RENCA tumor. *J. Immunol.* 161: 927-932
- ⁷⁶ Manome et al. 1995. Monocyte chemoattractant protein-1 (MCP-1) gene transduction: an effective tumor vaccine strategy for non-cranial tumors. *Cancer Immunol. Immunother.* 41: 227-235.
- ⁷⁷ Nakashima, E., Oya, A., Kubota, Y., et al. A candidate for cancer gene therapy: MIP-1 alpha gene transfer to an adenocarcinoma cell line reduced tumorigenicity and induced protective immunity in immunocompetent mice. *Pharm Res* 1996; 13: 1896-1901.
- ⁷⁸ Mule JJ, Custer M., Averbook B, et al. RANTES secretion by gene-modified tumor cells results in loss of tumorigenicity in vivo: role of immune cell subpopulations. *Hum Gene Ther* 1996; 7: 1545-1553.
- ⁷⁹ Hackett SP, Stevens DL. Superantigens associated with staphylococcal and streptococcal toxic shock syndrome are potent inducers of tumor necrosis factor-beta synthesis. *J Infect Dis* 1993; 168:232.
- ⁸⁰ Lienard D, Ewalenko P, Delmotte JJ, et al. High doses recombinant tumor necrosis factor alpha in combination with interferon gamma and melphalan in isolation perfusion of the limbs for melanoma and sarcoma. *J Clin Oncol* 1992; 10:52-60.
- ⁸¹ Smyth MJ, Pietersz GA, McKenzie IFC. Increased antitumor effect of immunoconjugates and tumor necrosis factor in vivo. *Cancer Res* 1988; 48:3607-12.