Chapter 15
Host Resistance Assays for Efficacy and Immunotoxicology Safety Evaluations

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Abstract The purpose of immunotoxicity testing is to obtain data that is meaningful for safety assessment and a number of approaches are available to achieve this goal. Regardless of the approach, it is important that the testing evaluate the three arms of the immune system and include assessments of innate immunity, CMI, and HMI.

When host resistance is used for immunotoxicity testing, immune suppression caused by a test compound or chemical may be reflected as impaired clearance of the infectious agent, increased sensitivity to opportunistic infections, prevention of immunization, or exacerbation of latent infections. In this immunotoxicity testing paradigm, the host resistance assay allows for an assessment of total functional immunocompetence and serves as a biomarker of net immunological health and well-being.

It is difficult to assess the biological relevance of an 'x' percent change in a particular immune parameter, such as reduction in thymus weight or reduction of NK activity, unless this change is evaluated in terms of overall biological impact. Host resistance assays, with the available redundancy inherent in the immune system, allow such an evaluation.

Comprehensive host resistance assays are used to evaluate the overall health of the immune system, i.e., how the numerous components of the functional immune system work together to clear an infection, while targeted host resistance assays are available to evaluate specific immunotoxicity questions. The choice of which host resistance assay to employ is dependent on the question being asked, and on the context of the question being posed (immunotoxicity or efficacy).

Keywords Immunotoxicity testing • Functional assays • Innate immunity • Cell-mediated immunity (CMI) • Humoral-mediated immunity (HMI) • IgM antibody • IgG antibody • Marginal zone B cells (MZB cells)

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15.1 Approaches to Evaluate Immunotoxicity

The purpose of immunotoxicity testing is to obtain data that is meaningful for safety assessment, the primary objective of which is to determine the significance of an immunotoxic effect with respect to immune suppression and increased susceptibility to infectious or neoplastic disease, or immune enhancement and hypersensitivity or autoimmunity.

Host resistance assays and animals models of disease are used as the principal method in evaluating the efficacy of antibacterial, antiviral and antifungal compounds during drug development. When host resistance is used for immunotoxicity testing, immune suppression caused by a test compound or chemical may be reflected as an impaired clearance of the infectious agent, increased sensitivity to opportunistic infections, prevention of immunization, or exacerbation of latent infections. In this paradigm, clearance of an infectious microorganism in a host resistance assay allows for an assessment of total functional immunocompetence and serves as a biomarker of net immunological health and well-being. The choice of which host resistance assay to employ is dependent on the question being asked, and also on the context of the question being posed (immunotoxicity or efficacy).

There are a number of approaches to evaluate immunotoxicity. A tiered approach is commonly used that evaluates standard toxicity tests followed by immunological function and host resistance.

15.1.1 Standard Toxicity Studies (STS)

Standard toxicity studies include hematology, clinical chemistry, gross pathology, organ weights and histology. The STS screening assays provide some initial information on the immune cells and organs that are effected by drug treatment or chemical exposure.

15.1.2 Functional Assays

Functional assays extend the findings in the STS screening assays and help determine if the STS effect on immune cells and organs translates into a defect in immune function. Functional assays should include a thorough evaluation of the three arms of the immune system: innate immunity, cell-mediated immunity (CMI), and humoral-mediated immunity (HMI).

Innate immunity, a nonspecific immune response involving immunological mediators that recruit and activate innate immune cells, is considered the first line of immune defense following immune challenge. The cytokine and chemokine

immunological mediators serve an important role in cell–cell communication among cells of the immune system, and call-in and activate immune system cells. The main cell types recruited and activated in the innate response include natural killer (NK) cells, macrophages, and neutrophils, among others. By virtue of their location throughout the body and their specialized functions, these cells facilitate a critical early interaction and are important contributors to early nonspecific innate immunity, and participate in specific immunological responses as well. Several assays exist to examine innate immune function. The measurement of NK cell activity, for example, is an important functional assay of innate immunity.

CMI is an adaptive immune response that involves antigen-specific recognition by T cells. Delayed type hypersensitivity (DTH) and cytotoxic T lymphocyte activity (CTL) are used to assess CMI in vivo. The CTL response requires the function and interaction of several categories of immune cells, including professional antigen-presenting cells such as dendritic cells and/or macrophages, $CD4^+$ T lymphocytes that produce help for response to T-dependent antigens, and $CD8^+$ T lymphocytes that develop into antigen-specific cytotoxic effector cells. In the context of host resistance, antigen-specific $CD8^+$ cytotoxic T lymphocytes (CTL) mediate the cell killing of intracellular pathogens or tumor cells (Burleson et al. 2010).

HMI is an adaptive immune response characterized by the production of antibodies including IgM and IgG. The antibody produced is important in preventing infection by infectious microorganisms, if previously immunized, or can be an important immunological therapeutic in limiting spread and eliminating infectious agents. The antibody response may be classified as a T-dependent antibody response (TDAR) or as a T-independent antibody response (TIAR).

The TDAR response requires and measures the functionality of three major immune cell types: T cells, B cells, and antigen presenting cells such as dendritic cells and macrophages. Measurement of TDAR is important in assessing the ability of the host to produce antibody. TDAR may be measured by evaluating the number of antibody forming cells (AFC) in the spleen following immunization with sheep red blood cells (SRBC). The SRBC AFC assay uses a technique of Jerne and Nordin (Jerne and Nordin 1963). TDAR may also be measured by immunizing with T dependent antigens such as keyhole limpet hemocyanin (KLH), tetanus toxoid, hepatitis B surface antigen, or SRBC and measuring the antigen specific antibody in the serum by ELISA. The ELISA method measures antibody in the serum from immunized animals and therefore results in a systemic evaluation of antibody production, rather than only in the spleen for the AFC method (White et al. 2007). Antibody class switching can also be evaluated in the serum if analysis includes both the IgM and the IgG antibody response.

The TIAR is important in recognizing polysaccharide antigens, such as those present on the encapsulated bacteria that cause bacterial pneumonia. This antibody response occurs in the absence of T cell help and requires the presence of marginal zone B cells (MZB cells) (Burleson and Burleson 2008a, b).

15.1.3 Host Resistance Assays (Animal Disease Models): The "Gold Standard"

Luster initiated a series of seminal studies that form the basis of risk assessment in immunotoxicology evaluations (Luster et al. 1988; Luster, Pait, et al. 1992; Luster, Portier, et al. 1992; Luster et al. 1995). These seminal studies evaluated various immunological assays that predicted immunotoxicity, and reported concordance values if the test material resulted in a dose-dependent response or altered two or more immune assays, as compared to host resistance assays. Since the primary responsibility of the immune system is to protect against infectious or neoplastic disease, host resistance assays are considered to be the ultimate predictor of adverse effects (Germolec 2004), and are the gold standard for immunotoxicological evaluation.

Many immunotoxicologists regard host resistance assays to be the most relevant for both validating the usefulness of other detection methods and for extrapolating the potential of a substance, drug or chemical, to alter host susceptibility in the human population (Germolec 2004). In general, host resistance assays determine if a drug or chemical results in an adverse effect (decreased immunity to the infectious agent), and provide potential mechanism(s) underlying the adverse effect (alterations related to cytokines, innate immune function, or adaptive immunity).

While mortality host resistance studies have been used more widely in the past, evaluation of immunity in host resistance models where mortality is not an endpoint is more consistent with current animal welfare concerns and the 3Rs. Challenging the immune system with an extremely virulent or with an extremely high titer of the infectious agent overwhelms the immune system with death occurring before development of the cascade of immunological responses required for clearance of the infectious challenge organism. Challenge in excess may reflect a model of sepsis or "cytokine storm" rather than a method to evaluate immunotoxicity. Immunological clearance of the infectious challenge agent is a more sensitive and meaningful measure of immunological function (Burleson 1995; Selgrade and Daniels 1995; Lebrec and Burleson 1994) than mortality. To evaluate bacterial clearance, the number of infectious particles per organ or per gram of organ is quantified at various time points following administration of the test article or chemical.

Immunological reserve can only be evaluated using host resistance assays. The immune response to an infectious agent results in the production of cytokines and chemokines, activation of neutrophils, macrophages and NK cells (innate immunity), activation of cell-mediated immunity (CMI), and lastly activation of humoral-mediated immunity (HMI). If, for example, there is some suppression of NK cell function, the functions of macrophages, neutrophils, CMI and HMI may be able to successfully combat the infection. The remaining functional components of the immune response thus function as the immunological reserve required to successfully combat the infection and can only be evaluated by use of host resistance assays.

With regard to the available models, the emphasis should not be placed on the particular viral, bacterial, or fungal agent that serves as the antigenic challenge, but rather on how the immune system responds to a challenge with a natural antigen.

Host resistance assays provide the only means to directly assess the functional reserve of the immune system, and may be classified as comprehensive host resistance assays or targeted host resistance assays. Comprehensive host resistance assays are used to evaluate the overall health of the immune system, i.e., how the numerous components of the functional immune system work together to clear an infection, while targeted host resistance assays are available to evaluate specific immunotoxicity questions.

15.2 Comprehensive Host Resistance Assay

15.2.1 Influenza Virus Host Resistance Assay

The influenza model in mice or rats is used to evaluate the overall health of the immune system (Table 15.1). Influenza virus host resistance models in mice and rats do not cause mortality, and are perhaps the most thoroughly characterized of all host resistance models. The clearance of influenza virus requires an intact and functional immune system that incorporates a cascade of immune responses and provides a measure of the health of the immune system. Mechanistic immune functions may be measured alongside viral clearance, and may include cytokine levels, macrophage activity, NK cell activity, CTL activity, and influenza-specific IgM and IgG. Measurement of these immunological functions provides an evaluation of innate immunity (cytokine, macrophage, and NK activity), an evaluation of CMI (CTL activity), and an evaluation of HMI (influenza-specific IgM or IgG). Additionally, measurement of influenza-specific IgM or IgG provides a measurement of the TDAR as influenza is a T-dependent antigen (Burleson 1995; Lebrec et al. 1996; Burleson 2000; Burleson and Burleson 2007, 2008a, b).

Table 15.1 Comprehensive host resistance assay

Influenza virus host resistance model
Evaluation of viral clearance as a measure of the integrated immune response
Additional mechanistic endpoints evaluated in this host resistance assay may include:
• *Histopathology*
• *Immunophenotyping and expression of surface markers on immune cells*
• *Cytokines and chemokines—immune mediators*
• *Macrophage activity—innate immunity*
• *NK cell activity—innate immunity*
• *CTL activity—cell-mediated immunity*
• *Influenza-specific antibody (IgM and IgG)—humoral-mediated immunity (TDAR)*

15.3 Targeted Host Resistance Assays

While the influenza host resistance assay is used to assess the overall health of the immune system, targeted host resistance models are available to answer specific questions concerning the nature of a potential immune deficiency (Table 15.2). Targeted host resistance assays may be used if a deficit has been identified, or if a deficit is of concern based on screening assays, functional assays, or data related to test articles with a similar chemical structure or mechanism of action. Targeted host resistance assays determine whether the specific immune deficit in question is adverse, i.e., does a percent decrease in a particular immunological parameter translate to a decreased clearance of the infectious agent.

15.3.1 Bacterial Host Resistance Assays

15.3.1.1 Evaluation of Neutrophil and/or Macrophage Function

Streptococcus pneumoniae Host Resistance Model

Rodent models for bacterial pneumonia have been used to evaluate the potential for immunotoxicity resulting from therapeutics targeting neutrophil and macrophage function. Macrophages were demonstrated to be important in the clearance of streptococci from the lungs of mice (Gilmour et al. 1993) and rats (Gilmour and Selgrade 1993). Further studies by Gilmour and Selgrade demonstrated the importance of neutrophils in pulmonary streptococcal disease in rats by pretreatment with an antibody to neutrophils. The *Streptococcus pneumoniae* host resistance model in rodents has been used in numerous immunotoxicity evaluations in BALB/c and C57BL/6 mice, as well as Fischer 344 (CDF), Lewis, and Sprague Dawley (CD) rats (Steele et al. 2005). In this model, rodents are infected intranasally, and bacterial clearance is measured and compared between test and control groups within 48 h, before the development of adaptive immune responses (Gilmour and Selgrade 1993; Gilmour et al. 1993; Burleson and Burleson 2006). Bacterial clearance is determined by

Table 15.2 Targeted host resistance assays

Host resistance assays for evaluation of specific immunotoxicity endpoints	
Evaluation of defect in neutrophil and/or macrophage function	*Streptococcus pneumoniae* pulmonary host resistance model *Pseudomonas aeruginosa* pulmonary host resistance model *Listeria monocytogenes* systemic host resistance model
Evaluation of immune effect of anti-inflammatory therapeutics	*Streptococcus pneumoniae* pulmonary host resistance model
Evaluation of immune effect of therapeutics targeting TNFα	*Streptococcus pneumoniae* pulmonary host resistance model
Evaluation of defect in Marginal Zone B (MZB) cell	*Evaluation of TIAR*—Systemic *Streptococcus pneumoniae* host resistance model to evaluate MZB cells

determining the number of colony forming units (CFU) per gram of lung tissue. Dexamethasone or cyclophosphamide is generally used as a positive immunomodulatory control as they have well-documented immunosuppressive effects on innate immunity and bacterial clearance. Cytokines may also be measured in the streptococcal model. Cytokines may be measured in the lung as well as in the serum. Bacterial titers and bacterial clearance are quantified as the number of colony forming units (CFU) per organ or per gram of tissue. Macrophage and/or neutrophil function assays may be measured as a mechanistic probe if an effect on bacterial clearance is observed. However, the conclusive observation is bacterial clearance. The *S. pneumoniae* host resistance model has been used in numerous therapeutic evaluations to evaluate the potential for pharmaceutical agents to induce neutrophil and/or macrophage immunotoxicity, and was reported as one of a battery of three host resistance assays to evaluate a small molecule therapeutic targeted for splenic tyrosine kinase (Syk) in mice (Zhu et al. 2007).

Pseudomonas aeruginosa Host Resistance Model

Pseudomonas aeruginosa is used as a pulmonary bacterial host resistance model to evaluate the immunotoxicity of therapeutics when an immunotoxic effect is suspected in neutrophils and macrophages (Gosselin et al. 1995). *Pseudomonas aeruginosa* a Gram negative bacillus that is a human pathogen that primarily causes diseases of the urinary tract, burn patients, septicemia, abscesses, corneal infections, meningitis, bronchopneumonia, and subacute bacterial endocarditis. Treatment often fails and the mortality rate in Pseudomonas septicemia has been reported to be greater than 80%. As in the *S. pneumoniae* pulmonary model, rodents are infected intranasally, and bacterial clearance is measured and compared between test and control groups within 48 h, before the development of adaptive immune responses. Bacterial clearance is determined by determining the number of CFU per gram of lung tissue.

Listeria monocytogenes Host Resistance Model

The *Listeria monocytogenes* host resistance model is a systemic infection assay that is used in the evaluation of adverse effects on neutrophils and Kupfer cells of the liver as well as splenic macrophages and neutrophils. NK cells and T lymphocytes also play a role in bacterial clearance. This host resistance model has been used to evaluate monoclonal antibodies directed against CD11b/CD18 (Mac-1), a leukocyte integrin that plays an important role in neutrophil adhesion and the acute inflammatory process and is a therapeutic anti-inflammatory target. CD11b (alpha M integrin) complexes with CD18 (beta 2 integrin) to form complement receptor type 3 (CR3) heterodimer. Treatment with either monoclonal antibody NIMP-R10 or 5C6, both directed against CD11b resulted in decreased clearance of listeria in the liver and spleen with increased mortality (Conlan and North 1992; Burleson and Burleson 2006). Both neutrophils and monocytes were decreased and mice were unable to control the infectious intracellular bacterial disease. Treatment of mice with a surrogate biological mAb designated NIMP-R10, directed against the CD11b polypeptide of the CD18/CD11b heterodimer, exacerbated listeriosis by preventing myelomonocytic cells from focusing at sites of infected hepatocytes in the liver. Under these conditions, an otherwise sub-lethal listeria inoculum grew unrestricted

within hepatocytes and caused death within 3 days (Conlan and North 1992). The results obtained with NIMP-R10 are similar to those reported with a different anti-CD11b mAb (5C6) (Rosen et al. 1989; Conlan and North 1991). The *Listeria monocytogenes* host resistance model is thus an important tool in evaluating the potential for disease enhancement and increased susceptibility to opportunistic infections in therapeutics or chemicals that interfere with bacterial clearance mechanisms.

15.3.1.2 Evaluation of Anti-Inflammatory Therapeutics

The *Streptococcus pneumoniae* pulmonary host resistance model in Lewis rats has been used to assess the effects of anti-inflammatory agents on innate immunity (Komocsar et al. 2007). While anti-inflammatory drugs are important to human health, it is known that these drugs are immunosuppressive with respect to innate immunity. The *S. pneumoniae* model is capable of measuring the suppression of the innate immune response following administration of anti-inflammatory test articles. The ability to rank order the severity of innate immune suppression with multiple test articles within the same study enhances the utility of this model for screening potential drug candidates.

15.3.1.3 Evaluation of Therapeutics Targeting TNF-α

The *S. pneumoniae* host resistance model is also valuable for evaluating the importance of macrophage cytokines on bacterial host resistance and for evaluating therapeutic agents that target TNF-α. In addition, this host resistance assay is useful in selecting a lead compound among compounds with equivalent therapeutic efficacy based on immunosuppression.

Human biological therapeutics targeting inhibition of TNF-α have been used to treat inflammatory autoimmune diseases such as rheumatoid arthritis, psoriasis, and Crohn's disease. Decreased TNF-α as a result of treatment with anti-TNF-α has an effect on several biomarkers of infection. Studies have reported that treatment of mice with a mAb to TNF-α results in altered levels of TNF-α in the lungs and serum, decreased neutrophils and increased numbers of bacteria (impaired bacterial clearance) with decreased survival in mice infected intranasally with *S. pneumoniae* (van der Poll et al. 1997; Takashima et al. 1997; Benton et al. 1998; O'Brien et al. 1999). The streptococcal pulmonary host resistance model is thus an important means to assess the functional immunological capacity of macrophages and neutrophils as well as macrophage cytokines.

15.3.1.4 Marginal Zone B (MZB) Cell Evaluation

Bacteria encapsulated with a polysaccharide capsule, such as *S. pneumoniae* or *Hemophilus influenzae*, are blood-borne pathogens that present a unique challenge to the immune system (Pillai et al. 2005). Capsular polysaccharide antigens are

thymus-independent type 2 antigens (TI-2) (Mond et al. 1995) and stimulate the TIAR, thus effective immune responses are dependent on the presence of a functional marginal zone (Amlot et al. 1985; Harms et al. 1996; Guinamard et al. 2000). The MZB cell model in mice or rats measures bacterial clearance, hematology, cytokine production, and antibody production at selected time points over a 14 day period after intravenous infection to create a blood-borne infection. MZB cells in both humans and rodents are considered a critical host defense mechanism directed against encapsulated blood-borne pathogenic microorganisms. Immunotoxicity directed against MZB cells not only decreases protection against blood-borne pathogens, but also results in a depletion of immunological memory. In summary, the TIAR is decreased or ablated as a result of MZB cell immunotoxicity (F.G. Burleson 2008).

Histopathology can detect defects in the splenic marginal zone and immunophenotyping markers can be included to detect alteration in the number of MZB cells. Should an effect on MZB cells be observed, the pharmaceutical agent may be evaluated in the *S. pneumoniae* systemic MZB host resistance model for encapsulated bacteria. The *S. pneumoniae* marginal zone B cell model has been characterized in mice and Sprague Dawley rats with a systemic blood-borne infection by intravenous inoculation. Bacteria are quantified by determining the number of CFU in the spleen, liver, lungs, and blood over a 2 week period. Cytokines, hematology, immunophenotyping, and anti-streptococcal antibody (TIAR) are also quantified in this model (Burleson and Burleson 2006).

15.3.2 Viral Reactivation Host Resistance Assay

Evaluating the potential for latent viral reactivation is critical, as reactivation of latent virus may result in a fatal disease such as progressive multifocal leukoencephalopathy (PML).

There are three major subfamilies of herpesviruses (alpha, beta and gamma), each containing strains susceptible to latent viral reactivation. These include the alpha herpesviruses family of herpes simplex virus-1 (HSV-1), HSV-2 and varicella-zoster virus (VZV); the beta herpesviruses that include human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV); and the gamma herpesviruses that include Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), and murine gammaherpesvirus-68 (MHV-68). Viruses capable of latent virus reactivation also include BK virus and JC virus. CMV (cytomegalovirus), EBV (Epstein-Barr Virus) and HSV (Herpes Simplex Virus) belong to the Herpesviridae virus family while BK virus and JC virus belong to the Papovaviridae virus family. While these viruses may belong to different families and subfamilies, there are many similarities between the viruses capable of causing latent/reactivated viral disease. All these viruses have double stranded DNA (the human polyoma viruses are circular); are ubiquitous in the human population; cause mild primary infections followed by a latent viral infection; and immunosuppression, especially a suppressed CMI results in reactivation of latent viral infection (G.R. Burleson 2008).

15.3.2.1 Murine Cytomegalovirus (MCMV) Latent Viral Model

The MCMV latent viral host resistance model can be used to evaluate immunotoxicity (Selgrade and Daniels 1995) or efficacy of antiviral therapies, as well as assess reactivation of latent viral disease due to immunosuppression (G.R. Burleson 2008). The MCMV reactivation model is useful when evaluating a pharmaceutical agent to determine if suppression of CMI or HMI results in reactivation of latent virus.

15.3.2.2 Murine Gamma Herpesvirus 68 (MVH-68) Latent Viral Reactivation Model

MHV-68 is a model for EBV, and both MHV-68 and EBV are lymphotropic, establish latency after acute lytic infection, and are associated with the development of lymphomas. A viral reactivation animal host resistance model with MHV-68 would allow the evaluation of immunosuppressive therapeutics for their ability to reactivate latent virus as measured by qPCR gene expression, plaque assay, and/or development of lymphomas (Mikkelsen et al. 2014; Aligo, Walker, et al. 2015; Aligo, Brosnan, et al. 2015).

15.3.3 Fungal Host Resistance

Candida albicans is a well-characterized fungal host resistance model in mice capable of evaluating complex interaction of innate and adaptive immunity, as both are important for recovery from Candidiasis infections, as well as evaluating immunotoxicity against fungal infections or efficacy of antifungal therapeutics. (Herzyk et al. 2001; Burleson and Burleson 2006). Ashman et al. and Netea et al. have reported on the immunological functions important for clearance of *Candida albicans* infections (Ashman et al. 2004; Netea et al. 2015). Both neutrophils and macrophages represent the first line of defense, while CMI with T cells is also essential for recovery from Candida infections.

In this model, Candida is administered intravenously, and clearance is evaluated on Days 4, 7, 14, and 21. The *Candida albicans* host resistance model allows an assessment of total immunocompetence by evaluating clearance of the organism. Clearance of a fungal infection requires an intact and functional immune system that incorporates a cascade of immune responses including: cytokine production, NK cell activity, macrophage activity, CTL activity and antibody production.

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