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EDITORIAL

Evaluation of virus-specific cellular immune response in transplant patients

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Abstract

Virus-specific immune responses have a major impact on the outcome of the infection. Viral agents that are characterized by latency, such as herpesviruses and polyomaviruses, require a continuous immune control to reduce the extent of viral reactivation, as viral clearance cannot be accomplished, independently from the anti-viral treatment. In transplant patients, morbidity and mortality related to viral infections are significantly increased. In fact, the key steps of activation of T-cells are major target for anti-rejection immunosuppressive therapy and anti-viral immune response may be altered when infected cells and cellular effectors of immune response coexist in a transplanted organ. The role of cellular immune response in controlling viral replication and the main methods employed for its evaluation will be discussed. In particular, the main features, including both advantages and limitations, of available assays, including intracellular cytokine staining, major histocompatibility complex - multimer-based assays, Elispot assay, and QuantiFERON test, will be described. The potential applications of these assays in the transplant context will be discussed, particularly in relation to cytomegalovirus and polyomavirus BK infection. The relevance of introducing viro-immunological monitoring, beside virological monitoring, in order to identify the risk profile for viral infections in the transplant patients will allows for define a patient-tailored clinical management, particular in terms of modulation of immunosuppressive therapy and anti-viral administration.

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INTRODUCTION

Virus-specific immune responses have a major impact on the outcome of the infection. Viral infections are usually followed by the complete clearance of the virus. However, in some cases, immune response is not able to eliminate completely the virus, thus the infection may become persistent. In general, cellular immune response plays a more relevant role in the viral clearance, whereas humoral immune response protects against the reinfection. Immune response may vary according to genetic substrate of the individual and the degree of immunocompetence.

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mune functions and anti-viral immune response may be altered when infected cells and cellular effectors of immune response coexist in a transplanted organ.

In particular, viral agents that are characterized by latency, such as herpesviruses and polyomaviruses, require a continuous immune control to reduce the extent of viral reactivation, as viral clearance cannot be accomplished, independently from the anti-viral treatment. The immunologic control of these viruses in the immunocompromised host is complex and involves both the innate and adaptive immune systems. As regards innate immune system, polymorphisms of Toll-like receptors as well as certain proteins, including complement proteins, and defects in natural killer cells are associated with an increased risk of viral reactivation. Adaptive immune responses of B and T cells are critical in controlling viral replication. In particular, while B cells may be important in the humoral response in that they produce neutralizing antibodies targeting virions, T-cell mediated responses, including both CD4+ and CD8+ T-cells, are crucial components for controlling viral replication of persistent viruses[1]. Several phenotypic markers and functions have been investigated in order to characterize virus-specific CD4+ and CD8+ T cell responses, evidencing a great heterogeneity against different viruses, with functional signatures being probably specific for each virus and being predominantly regulated by the levels of antigen load. In this context, polyfunctional secretion and proliferation [i.e., interferon (IFN)-γ and interleukin-2] of CD4+ and CD8+ T cells play a protective role in terms of antiviral immunity in chronic viral infections[2,3].

METHODS FOR EVALUATING VIRUS-SPECIFIC CELLULAR IMMUNE RESPONSE

Evaluation of virus-specific T-cell responses can be made by different methods (Table 1), including: intracellular cytokine staining (ICS), major histocompatibility complex (MHC)-multimer-based assay, Enzyme-linked Immunospot (ELISPOT) assay and QuantiFERON-cytomegalovirus (CMV) assay (specific for CMV)[4-6]. Beside these virus-specific assays, it is also available a non-specific test, the ImmunKnow assay, that is used to evaluate the overall CD4+ immune response.

Most of these assays have been used in experimental settings and only for some of them commercial kit are available. The majority of the assays are based on the detection of IFN-γ after stimulation with specific viral antigens or viral lysates, the so-called IFN-γ releasing assays (IGRA). Anyway, other markers, including different cytokines (e.g., interleukin-2, tumor necrosis factor-α, *etc.*) can be used. In terms of clinical utility, an ideal test should evaluate both virus-specific CD4+ and CD8+ T-cell immune response from both quantitative (number of virus-specific T cells) and function (number of functional T cells) points of view. Moreover, for an appropriate use in the clinical setting, an assay should be easy and rapid to perform, relatively inexpensive, highly reproducible, and

applicable in different routine contexts. At the moment, all the assays present specific advantages and limitations.

Intracellular cytokine staining
Most studies have analyzed virus-specific T-cell responses using ICS for IFN-γ using flow cytometry[4]. Moreover, ICS allows for evaluation of polyfunctionality of T cells[2,3]. Whole blood or isolated peripheral blood mononuclear cells (PBMCs) are stimulated with virus-specific peptides or viral lysates. The assay is not restricted for human leukocyte antigen (HLA) when viral lysate is used and there Is no need to know HLA type. Stimulated cells are stained with monoclonal antibodies directed against IFN-γ. The method is rapid, with a short incubation time and results being available within 24 h; can be performed starting from low blood volume (approximately 1 mL); it allows for identification of CD4+ and CD8+ T cells. The major drawbacks are the need for a flow cytometer and the lack of standardization. Among the advantages, there is the possibility to freeze the cells and send them to reference laboratory for testing.

MHC - multimer-based assays
MHC-multimer-based assays is characterized by the direct staining of peptide-specific T-cells using peptide-conjugated MHC class I tetramers or pentamers[7]. This method evaluates CD8+ T-cell responses, however it is epitope-specific and require knowledge of the patient's HLA type. As for ICS, there is the need for access to a flow cytometer and the assay is not standardized. Among the advantages, the assay is rapid (1-2 h) and can be performed starting from low blood volume (0.5-1 mL).

Elispot
The ELISPOT assay determines the number of T-cells secreting IFN-γ in specific response to a viral agent. Following stimulation with viral peptides or viral lysates, the produced IFN-γ is captured by a specific antibody, and then quantified using a labeled antibody[8]. The assay evaluates both CD4+ and CD8+ responses and there is no need to know HLA, as well as to use a flow cytometer. There is the possibility to freeze the cells and send them to reference laboratory for testing. Among the diasadvantages, the method requires a relatively high volume of blood (approximately 7-10 mL) and cannot differentiate between CD4+ and CD8+ cells. Although some approaches to standardize the assay, it is still not standardized. Results are available within 24-30 h. A mitogen control assay can determine general T-cell responsiveness. Commercially available kits for virus-specific ELISPOT assays are available (AID, Autoimmun Diagnostika GmbH, Strassberg, Germany).

QuantiFERON-CMV
This is a CMV-specific ELISA-based IFN-γ release assay and is commercially available (Cellestis Inc., Melbourne, Australia). It can be easily performed starting from low blood volume (3 mL) and results are available after

Table 1 Methods for evaluating cellular immune response

Method	Advantages	Limitations
Intracellular cytokine staining	Low volume blood	Flow cytometer required
	Rapid	Not standardized
	Knowledge of HLA not required	
	Identification of CD4+ and CD8+	
MHC multimer staining	Low volume blood	Flow cytometer required
	Rapid	Not standardized
Elispot assay	Identification of CD4+ and CD8+	Not standardized

MHC: Major histocompatibility complex; HLA: Human leukocyte antigen.

30-40 h. It evaluates only CD8+ responses. The assay may yield nonresponse results in the presence of global immunosuppression (nonresponse to mitogen) as in transplant patients. Test sensitivity decreases in lymphopenic patients because an appropriate number of T-cells are required for the production of IFN-γ.

ImmunKnow assay

The Cylex ImmunKnow assay (Cylex Inc., Columbia, MD, US) is a aspecific assay, which is commercially available. It measures the overall immune response and is indicative of immunesuppression. It determines the amount of ATP produced in response to whole-blood stimulation by an aspecific mitogen (phytohemagglutinin).

ROLE OF VIRUS-SPECIFIC CELLULAR IMMUNE RESPONSE IN TRANSPLANT PATIENTS

T-cell responses, including both CD4+ and CD8+, are critically important for controlling viral replication. This has been demonstrated by the use of adoptive immunotherapy for treatment of CMV and EBV infections and by the higher frequency of viral reactivation in patients treated with anti-lymphocyte agents. Early reconstitution of cellular immune response prevents or reduces the duration of the infection, thus avoiding the onset of disease or relapses. On the other hand, a delayed or reduced response represents the pathogenic base for the occurrence of repeated episode of infection and symptomatic disease (in the absence of anti-viral treatment)[7-11]. The role of cellular immune response in the context of organ transplantation has been studied particularly for CMV. In particular, it has been evidenced the basic role played by both CD4+ and CD8+ responses[9]. The CD8+ response is prevalent during the acute phase of infection and it provides for an immediate control of viral replication by the killing of cells in which CMV is replicating. The CD4+ response is fundamental for the long-term maintenance of antiviral control. In a study on solid-organ transplant recipients, it has been demonstrated that stable levels of CMV-specific CD4+ cells correlates with the absence of infectious complications, whereas in patients with unstable levels of specific-CD4+ cells, several episodes of viral reactivation occurred[10]. The median

frequency of CMV-specific CD8+ cells was significantly higher in a group of 27 heart and lung transplant patients in cases that did not developed CMV-disease[11]. Moreover, in a study on 73 renal transplant recipients, also the median frequency of CMV-specific CD4+ cells was significantly higher in patients that did not developed CMV disease[12,13]. Gerna and coll. proposed the classification of transplant recipients into two groups in relation to the temporal profile for CMV-specific cellular immune response[14]. The Authors considered a group of early responders, in which reconstitution of cellular immune response occurred within 30 d posttransplantation, and a group of late responders, in which cellular immune response reconstitution occurred at > 30 d posttransplantation and/or was reduced. In these patients, the delay in CD4+ response appeared to be particularly critical[15].

Another virus for which specific cellular response has been investigated is polyomavirus BK. It has been evidenced that healthy BKV-seropositive individuals have CD4+ and CD8+ cells specific for BKV major antigens (including large T antigen and capsid viral protein VP1)[16]. The unbalance between viral replication and BKV-specific cellular immune response should represent the common denominator for the pathogenesis of polyomavirus-associated nephropathy, a viral-associated complication potentially leading to the lost of the transplanted organ and mainly occurring in renal transplant patients[15,16]. It has been evidenced that cellular response to large T antigen and VP1 is significantly higher in patients with decreasing viremia in comparison to those with increasing viremia[16]. Moreover, it seems that the level of immune response is correlated particularly to the administration of some immunosuppressive drugs. For example, in a study on kidney transplant patients *in vivo*, responses were inversely correlated with tacrolimus through levels, but not mycophenolate mofetil, prednisone or the overall immunosuppressive dosing[17]. However, the clinical role of BKV viro-immunological monitoring in renal transplant recipient needs to be further investigated, in particular it is likely that it could represent an approach to modulate immunosuppression.

An interesting issue in the context of protective immunity is represented by the role of mucosal immunization. As mucosal surfaces represent the major entry fro many human pathogens (including HSV, HIV, respiratory viruses, as well as mycobacteria), induction of mucosal

immune system, including both innate and adaptative responses (CD4+ T helper cells, Th17 cells, high avidity CD8+ cytotoxic T lymphocytes, as well as IgA and IgG1 neutralizing antibodies) seem required for an effective protection against pathogens that lead to chronic infections[18,19].

CONCLUSION

The increase in the number of transplant patients and the use of deeply immunosuppressive drugs have lead to the emergence of viral infections that may influence the outcome of these patients. Beside the adoption of protocols for close virological monitoring, several studies have underlined the role of viro-immunological monitoring in the evaluation of patient's risk of infection and disease, decision on treatment by modulating immunosuppression and/or using antiviral, and adoption of other diagnostic strategies. There is no defined method to evaluate the cellular immune response in transplant recipients, as at the moment no assay is standardized and further studies, particularly on procedures, quality controls and references, as well as interpretation of results are required to standardize. However, ELISPOT assay is gaining increasing recognition due to the potential to evaluate both CD4+ and CD8+ responses and other favoring features. Further studies on the clinical role of cellular immune responses are required, in particular with the aim of define modes and temporal profile for viro-immunological monitoring in different transplant contexts and for different viral agents.

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