# Development of a coordinated allo T cell and auto B cell response against autosomal PTK2B after allogeneic hematopoietic stem cell transplantation

Anita N. Kremer,[1,2] Judith C. van der Griendt,[1] Edith D. van der Meijden,[1] M. Willy Honders,[1] Burcu Ayoglu,[3] Jochen M. Schwenk,[3] Peter Nilsson,[3] J.H. Frederik Falkenburg,[1] and Marieke Griffioen[1]

[1]Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands; [2]Department of Internal Medicine 5, Hematology and Oncology, University of Erlangen, Germany; and [3]Science for Life Laboratory, KTH - Royal Institute of Technology, Solna, Sweden

## ABSTRACT

It is well known that allo-reactive T cells play a crucial role in graft-*versus*-leukemia and graft-*versus*-host disease after allogeneic hematopoietic stem cell transplantation (alloSCT). Allo-reactive CD4$^+$ T cells can mediate direct cytolysis, but may also stimulate production of IgG antibodies as helper cells. Immune complexes may subsequently be processed and presented by professional antigen presenting cells and stimulate induction of specific CD8$^+$ T cells. As such, proteins targeted in coordinated T- and B-cell responses may represent a class of immunodominant antigens in clinical responses after alloSCT. We previously identified LB-PTK2B-1T as HLA class II restricted polymorphic antigen in a patient treated with donor lymphocyte infusion for relapsed chronic myeloid leukemia after HLA-matched alloSCT. Since PTK2B has also been described as antibody target, we here investigated whether a coordinated T- and B-cell response against PTK2B was induced. Patient serum before and after alloSCT and donor lymphocyte infusion (DLI) was screened for antibodies, and we indeed observed development of a humoral immune response against PTK2B. Antibodies against PTK2B were only found after DLI and, in contrast to the CD4$^+$ T cells, recognized a monomorphic region of the protein. To our knowledge, this is the first description of a coordinated allo-reactive CD4$^+$ T-cell and auto-reactive antibody response against an autosomal antigen.

## Introduction

Allogeneic hematopoietic stem cell transplantation (alloSCT) is often the only therapeutic option for patients with hematologic malignancies. During this treatment, patient hematopoiesis is eradicated by high-dose radio-/chemotherapy and the patient is rescued by infusion of hematopoietic stem cells of an (HLA-matched) donor.[1] While in the early days of alloSCT, infusion of donor hematopoiesis was solely meant to rescue the patient from the otherwise lethal radio-/chemotherapy, it is now known that its beneficial effect is actually mediated by an immune response of donor cells against residual malignant cells of the patient: the so-called graft-*versus*-leukemia (GvL) effect.[2] The same immune reaction, however, can also be directed against healthy non-hematopoietic tissues of the patient causing detrimental graft-*versus*-host disease (GvHD).[3]

It is well established that beneficial GvL effect as well as undesired GvHD are mediated by donor-derived allo-reactive T cells. While CD8$^+$ T cells confer GvL effect and GvHD via their direct cytolytic activity, CD4$^+$ T cells might have a more diverse role. Although they can exert direct cytolytic activity, CD4$^+$ T cells are generally believed to play a central role as helper cells in immune responses.[4,5] CD4$^+$ T cells help by inducing maturation of dendritic cells and stimulation of CD8$^+$ T cells,[6] and they are also known to deliver essential signals for B cells to produce IgG antibodies.[7] Although the capacity of CD4$^+$ T cells to stimulate production of antibodies is well-known, and induction of auto- and allo-reactive antibodies after HLA-matched alloSCT has been demonstrated by various studies,[8-15] the link between CD4$^+$ T- and B-cell immunity after alloSCT has only been demonstrated for DDX3Y, a male specific antigen encoded on the Y-chromosome.[16,17]

We previously identified 5 HLA class II restricted polymorphic antigens as targets for allo-reactive CD4$^+$ T cells in a patient with relapsed chronic myeloid leukemia who responded to donor lymphocyte infusion (DLI) after HLA-matched alloSCT.[18,19] One of these antigens has been shown to be derived from human PTK2B, which is a member of the focal adhesion kinase family of proteins. PTK2B, also known as PYK2 or RAFTK, has previously been described as target for antibodies in 3 of 19 patients treated with DLI for relapsed CML after HLA-matched alloSCT.[10] However, whether these antibodies were allo- or auto-reactive and whether T cells specific for PTK2B were induced in these patients has not been addressed.

We investigated whether allo-reactive CD4$^+$ T cells against PTK2B exerted helper function *in vitro* and whether a coordinated B-cell response was induced *in vivo*. Allo-reactive CD4$^+$ T cells against LB-PTK2B-1T were shown to induce DC maturation and B-cell activation *in vitro*. Moreover, we showed *in vivo* induction of a humoral immune response against PTK2B after DLI.

 doi:10.3324/haematol.2013.086652
The Online version of this paper contains a Supplementary Appendix.
Manuscript received on March 1, 2013. Manuscript accepted on September 27, 2013.
Correspondence: m.griffioen@lumc.nl

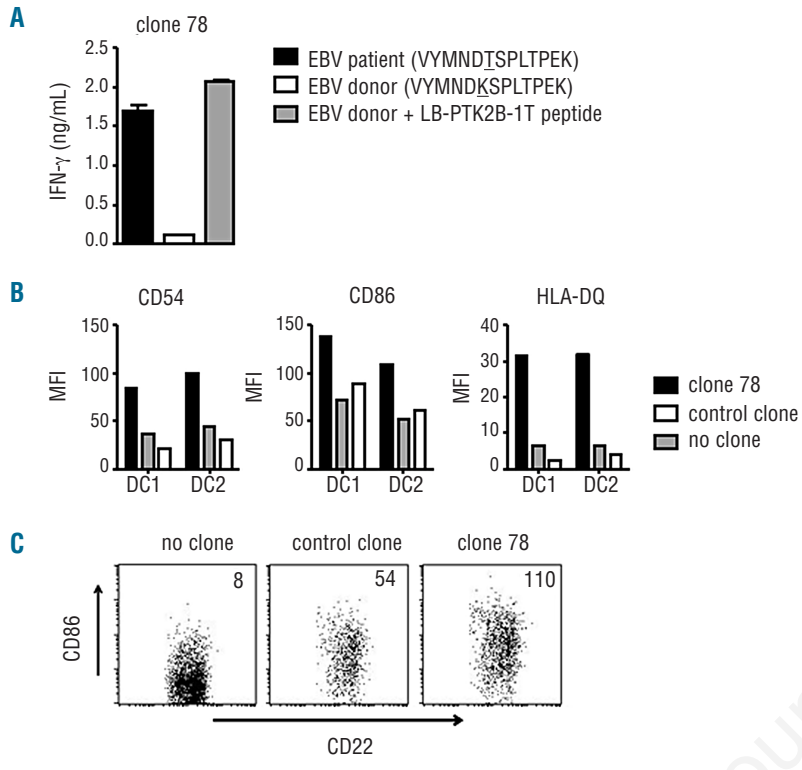


**Figure 1.** LB-PTK2B-1T specific CD4+ T cells can provide specific help. (A) LB-PTK2B-1T specific T-cell clone 78 has been isolated by flowcytometry based on expression of activation marker HLA-DR and CD4 from a bone marrow sample obtained five weeks after DLI from a patient with relapsed CML after HLA-matched alloSCT. The T-cell clone strongly recognized patient, but not donor, EBV-LCL as well as donor EBV-LCL loaded with LB-PTK2B-1T peptide in IFN-γ ELISA. Mean and standard deviation of 50-μL supernatants in duplicate wells is shown. (B) Immature DC of 2 LB-PTK2B-1T positive donors expressing HLA-DRB3*01:01 were co-cultured in the absence (open bars) or presence of CD4+ T-cell clone 78 (filled bar) or a CD4+ T-cell control clone (gray bar) specific for another previously identified HLA-DR restricted MiHA which is not expressed by the DC. Maturation of DC was assessed by measuring surface expression of CD54, CD86 and HLA-DQ after co-culture for 4 days. (C) LB-PTK2B-1T positive B cells expressing HLA-DRB3*01:01 were isolated and analyzed by flowcytometry after 2 days of culture in the absence (left) or presence (right) of CD4+ T-cell clone 78 or a CD4+ T-cell control clone (middle). Activation of B cells was measured by surface expression of CD86 and mean fluorescence intensities for gated B cells are depicted.

This antibody response was mapped to a small non-polymorphic region of the protein. In conclusion, this is the first report demonstrating induction of a coordinated allo-T cell and auto-B cell response against an autosomal antigen after alloSCT.

## Methods

### Hematopoietic samples

Peripheral blood and bone marrow samples were obtained from patients and healthy individuals after approval by the Leiden University Medical Center Institutional Review Board and informed consent according to the Declaration of Helsinki.

### Antigen presentation assays

EBV-LCL were loaded with 1 μg/mL of synthetic peptides in IMDM with 2% FCS and incubated for 2 h at 37°C. Cells were washed twice and plated at 30,000 cells/well in a 96-well plate. Effector cells were added at 5000 T cells/well. After overnight co-incubation, IFN-γ production was measured by ELISA (Sanquin, Burton upon Trent, UK) according to the manufacturer's instructions.

### Helper function assays

To induce maturation of dendritic cells (DC), CD4+ T-cell clones and immature DC were seeded in a 1:1 ratio in a 24-well plate. After four days of co-incubation, cells were harvested, washed and stained with antibodies for FACS analysis. For induction of B-cell activation, CD4+ T-cell clones and isolated B cells were seeded in a 1:1 ratio and after two days of co-incubation, cells were harvested, washed and stained for FACS analysis.

### Western blot analysis

Whole cell lysates of transfected HEK293T cells were obtained from OriGene (Rockville, USA). HeLa cells were retrovirally transduced with MP71 vector containing PTK2B or PI4K2B constructs; 20 μg of protein was loaded on each lane. SDS-Page was run on pre-cast NuPage® Novex 10% Bis-Tris Mini gels (Invitrogen) for 35 min at 30V under reducing conditions. Gels were blotted on PVDF membranes using XCell SureLock® Mini-Cell blotting system (Invitrogen) according to the manufacturer's instructions. Blots were blocked for 1 h at room temperature in phosphate-buffered saline with 0.05% Tween-20 and 5% BSA, and subsequently incubated with diluted (1:40) serum samples overnight at 4°C. Subsequently, the membrane was incubated with biotinylated anti-human IgG and streptavidin-QDots 625 (Invitrogen) for 1 h each and visualized under UV illumination.

### Suspension bead array

The suspension bead array was performed as previously described.[20,21] Purified proteins (20 μg) were coupled to carboxylated beads (Bio-Rad Laboratories B.V.) according to the manufacturers' instructions. Diluted serum samples (1:100 and 1:300) were pre-absorbed with 0.5% (w/v) polyvinylalcohol and 0.8% (w/v) polyvinylpyrrolidone (Sigma) (PVX) and 1% bovine serum albumin (BSA) for 1 h prior to incubation with the protein-coupled bead mix at room temperature on a shaker for 1 h. In specific blocking experiments, serum samples were pre-absorbed with purified recombinant proteins at 0.5 or 2.0 μg/mL in PVX and 1% BSA. Beads were washed and incubated for another hour with PE-labeled anti-human IgG, and fluorescence was measured on a Bioplex-100 (BioRad).

### Real-time polymerase chain reaction

Clonotypic real-time polymerase chain reaction (RT-PCR) was

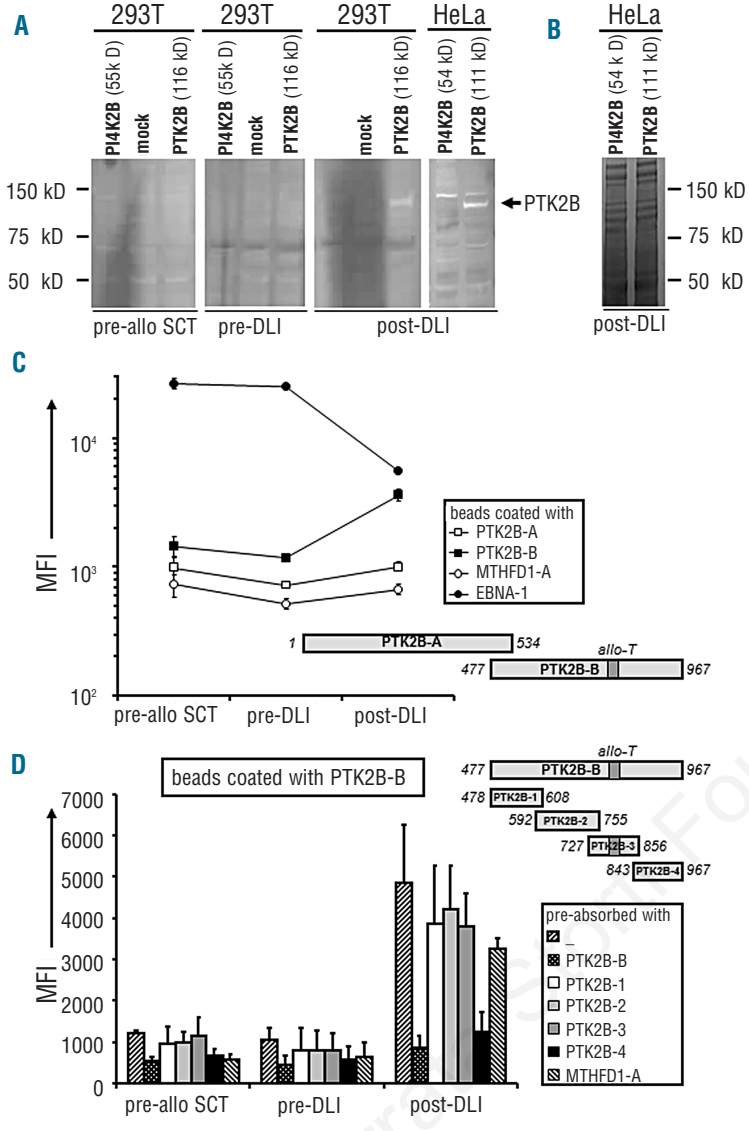


**Figure 2.** Detection of induced PTK2B specific auto-antibodies after donor lymphocyte infusion. (A) Western blots containing whole cell lysates of HEK293T cells transfected with PI4K2B, PTK2B or empty vector (mock) were incubated with diluted (1:40) serum samples from patient MRJ obtained before aSCT (left), after aSCT / before DLI (middle) and after DLI (right). The binding of serum antibodies was visualized by a secondary antibody against human IgG. HeLa cells retrovirally transduced with PI4K2B or PTK2B vector were incubated with diluted serum from patient MRJ obtained after DLI to confirm specific binding of serum antibodies to PTK2B. (B) As a control for protein loading, SDS-PAGE with HeLa cells transduced with PI4K2B or PTK2B were stained with Coomassie Brilliant Blue. (C) Multiplexed analysis of antibody recognition sites was conducted with recombinant proteins PTK2B-A (aa 1-534) and -B (aa 477-967) comprising the N- and C-terminal part of PTK2B, respectively, and EBV-derived antigen EBNA1 as positive control and the N-terminal part of MTHFD1 (MTHFD1-A) as negative control. Protein fragments were isolated, purified and coupled to color-coded suspension beads. Subsequently, serum samples (1:300) were incubated with protein-coated beads and binding of specific antibodies was measured by staining with PE-labeled anti-human IgG. Mean fluorescence intensities of single measurements of 2 independent experiments are shown. Indicated is the allo-T cell epitope at aa 791-800 in PTK2B-B. (D) To further map for the antibody epitope of PTK2B, inhibition assays were performed by adding soluble recombinant protein fragments as competitors. Serum samples (1:100 and 1:300) were pre-absorbed with recombinant PTK2B fragments 1 (aa 478-608), 2 (aa 592-755), 3 (aa 727-856), 4 (aa 843-967) or PTK2B-B (aa 477-967) prior to incubation with protein-coated beads. MTHFD1-A was included as negative control. Mean fluorescence intensities of single measurements with suspension beads coated with PTK2B-B of 2 independent experiments are shown. Inhibition of mean fluorescence intensity after pre-absorption with PTK2B-B and fragment 4 indicates specific binding of antibodies. Pre-absorption with these fragments did not inhibit antibody binding to EBNA-1 coated beads (*data not shown*).

designed with a forward primer specific for the variable T-cell receptor β-chain and a reverse primer specific for the CDR3 region of clone 78. Expansion of the PCR product was followed by measuring fluorescence of the DNA-intercalating fluorophore EvaGreen (Biotium, San Francisco, CA, USA), and specificity of the PCR product was confirmed by melting curve analysis.

## Results and Discussion

### *LB-PTK2B-1T specific CD4⁺ T cells can provide specific help*

LB-PTK2B-1T specific CD4⁺ T-cell clone 78 has been isolated 5 weeks after DLI based on expression of activation marker HLA-DR on bone marrow cells from a patient with relapsed CML after HLA-matched alloSCT.[19] The specificity of this T-cell clone was demonstrated by IFN-γ production upon stimulation with patient, but not donor, EBV-LCL as well as donor EBV-LCL loaded with LB-PTK2B-1T peptide (Figure 1A). To investigate *in vivo* dynamics of the T-cell response, we developed a clonotypic PCR for the CDR3 region of clone 78 and detected a specific PCR product in patient PBMC 9 weeks after DLI (Ct = 36), but not in patient samples collected before alloSCT or prior to DLI and in a donor sample, whereas the PBGD housekeeping gene was similarly expressed in all samples (Ct = 25-27) (*data not shown*). These data demonstrate that LB-PTK2B-1T specific CD4⁺ T cells specifically expanded *in vivo* after treatment with DLI. To address the helper potential of PTK2B-specific CD4⁺ T cells, we analyzed whether clone 78 was capable of inducing maturation of dendritic cells and activation of B cells in an antigen specific manner. We isolated CD14⁺ monocytes from PBMC endogenously expressing LB-PTK2B-1T and the HLA-DRB3*01:01 restriction allele, and subsequently differentiated these monocytes into immature dendritic cells in the presence of GM-CSF and IL-4. After 4 days of co-culture with PTK2B-1T specific CD4⁺ T-cell clone 78, the dendritic cells acquired a mature phenotype as measured by upregulation of HLA-DQ, costimulatory molecule CD86 and adhesion molecule CD54 (Figure 1B). Maturation of dendritic cells could not be measured after co-culture with a control T cell clone. In addition, we

isolated CD19+ cells and measured specific B-cell activation by upregulation of activation marker CD86 after 2 days of co-culture with T cell clone 78 (Figure 1C). In conclusion, the experiments show that PTK2B specific CD4+ T-cell clone 78 is a bona fide T helper cell as illustrated by its ability to induce DC maturation and B-cell activation *in vitro*.

### Induction of PTK2B specific auto-antibodies after donor lymphocyte infusion

To address the *in vivo* helper function of PTK2B-1T specific CD4+ T cells in more detail, we analyzed whether a co-ordinated T- and B-cell response against this protein was induced after DLI. Patient serum obtained before alloSCT, between T-cell depleted alloSCT and DLI, and after DLI were screened for anti-PTK2B IgG antibodies by Western blot analysis (Figure 2A and B) and multiplexed suspension bead arrays (Figure 2C and D), and antibody reactivity against PTK2B could be measured by both methods. Whereas antibody reactivity against CMV or EBV antigens was detectable in all serum samples, reactivity against PTK2B specifically evolved after DLI, and was not found before alloSCT or prior to DLI. Antibody reactivity against PTK2B after DLI could not be explained by an overall increase in antibodies after immune recovery, as witnessed by decreased binding of antibodies to EBV-derived antigen EBNA1 and lack of reactivity against other cellular proteins on Western blot. To further determine the immunogenic region of PTK2B, we produced partially overlapping recombinant protein fragments and mapping for recognition sites showed that antibody reactivity was directed against the C-terminal part of PTK2B (PTK2B-B) (Figure 2C). Further fragmentation of the C-terminus of PTK2B revealed that the antibody epitope was located in a non-polymorphic region between amino acids (aa) 843 - 967 (PTK2B-4) (Figure 2D), whereas the polymorphic T-cell epitope is located between aa 791 - 800. By sequencing PTK2B cDNA of patient and donor, we confirmed that no differences were present within this region (*data not shown*), illustrating that the humoral immune response after DLI consisted of auto-antibodies against PTK2B.

### Conclusions

In this report, we demonstrate induction of a co-ordinated allo-CD4+ T cell and auto-B cell response against autosomal antigen PTK2B after alloSCT. In contrast to the coordinated B- and T-cell response against DDX3Y in which an allo-reactive antibody specific for the protein variant encoded on the Y-chromosome has been shown to co-develop with an auto-reactive CD4+ T cell recognizing both protein variants encoded on the Y- and X-chromosomes,[16,17] we demonstrate induction of an allo-reactive CD4+ T cell specific for a polymorphic epitope combined with an antibody directed against a monomorphic region of the same protein. It has been shown that approximately 20% of the mature peripheral B-cell repertoire is self-reactive,[22,23] but that the majority of B cells are anergic to prevent production of auto-antibodies.[24,25] Self-tolerance can be broken by molecular mimicry, in which antibodies generated in response to an infectious agent are cross-reactive with self proteins. In these cases, antibody responses are usually transient as the involved T cells are directed against foreign pathogen-derived antigens and not against self-proteins. In the context of DLI for leukemic relapse, it is conceivable that release of cytosolic contents due to tissue damage mediated by activated allogeneic T cells may have triggered a break in B-cell tolerance and production of auto-antibodies against intracellular self-antigens.[10] Although antibodies against intracellular antigens are not expected to mediate direct cytolysis, immune complexes may be formed, which can be efficiently processed and presented by professional antigen presenting cells, thereby stimulating induction or amplification of specific T-cell responses.[26-28] As such, proteins targeted in coordinated T- and B-cell responses may represent a class of immunodominant antigens in clinical responses after alloSCT. However, it should be noted that, although simultaneous development of T- and B-cell responses against the same protein is suggestive for cognate T-cell help, non-cognate T-cell responses and direct stimulation by TLR-ligands cannot be ruled out, and may also be considered as potential stimulators for auto-reactive B cells.

In conclusion, our data demonstrate development of a co-ordinated CD4+ T- and B-cell response against autosomal antigen PTK2B. Based on this patient case, it is tempting to speculate that allo-reactive CD4+ T cells as administered by DLI may break B-cell tolerance and stimulate development of auto-antibodies after alloSCT.

### Funding

*This work has been supported by the Dutch Cancer Society (UL2008-4111) and the German Research Foundation (DFG) (STU540/1-1).*

### Authorship and Disclosures

*Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.*

## References

1. Copelan EA. Hematopoietic stem-cell transplantation. N Engl J Med. 2006;354(17):1813-26.
2. Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. Blood. 1995;86(5):2041-50.
3. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. Lancet. 2009;373(9674):1550-61.
4. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. Nature. 2003;421(6925):852-6.
5. Toes RE, Schoenberger SP, van der Voort EI, Offringa R, Melief CJ. CD40-CD40Ligand interactions and their role in cytotoxic T lymphocyte priming and anti-tumor immunity. Semin Immunol. 1998;10(6):443-8.
6. Zorn E, Wang KS, Hochberg EP, Canning C, Alyea EP, Soiffer RJ, et al. Infusion of CD4+ donor lymphocytes induces the expansion of CD8+ donor T cells with cytolytic activity directed against recipient hematopoietic cells. Clin Cancer Res. 2002;8(7):2052-60.
7. MacLennan IC. Germinal centers. Annu Rev Immunol. 1994;12:117-39.
8. Wadia PP, Coram M, Armstrong RJ, Mindrinos M, Butte AJ, Miklos DB. Antibodies specifically target AML antigen NuSAP1 after allogeneic bone marrow transplantation. Blood. 2010;115(10):2077-87.
9. Marina O, Hainz U, Biernacki MA, Zhang W, Cai A, Duke-Cohan JS, et al. Serologic markers of effective tumor immunity against chronic lymphocytic leukemia include nonmutated B-cell antigens. Cancer Res. 2010;70(4):1344-55.

10. Wu CJ, Yang XF, McLaughlin S, Neuberg D, Canning C, Stein B, et al. Detection of a potent humoral response associated with immune-induced remission of chronic myelogenous leukemia. J Clin Invest. 2000;106(5):705-14.
11. Bellucci R, Alyea EP, Chiaretti S, Wu CJ, Zorn E, Weller E, et al. Graft-versus-tumor response in patients with multiple myeloma is associated with antibody response to BCMA, a plasma-cell membrane receptor. Blood. 2005;105(10):3945-50.
12. Miklos DB, Kim HT, Miller KH, Guo L, Zorn E, Lee SJ, et al. Antibody responses to H-Y minor histocompatibility antigens correlate with chronic graft-versus-host disease and disease remission. Blood. 2005;105(7):2973-8.
13. Biernacki MA, Marina O, Zhang W, Liu F, Bruns I, Cai A, et al. Efficacious immune therapy in chronic myelogenous leukemia (CML) recognizes antigens that are expressed on CML progenitor cells. Cancer Res. 2010;70(3):906-15.
14. Biernacki MA, Tai YT, Zhang GL, Alonso A, Zhang W, Prabhala R, et al. Novel myeloma-associated antigens revealed in the context of syngeneic hematopoietic stem cell transplantation. Blood. 2012;119 (13):3142-50.
15. Pfistershammer K, Lawitschka A, Klauser C, Leitner J, Weigl R, Heemskerk MHM, et al. Allogeneic disparities in immunoglobulin-like transcript 5 induce potent antibody responses in hematopoietic stem cell transplant recipients. Blood. 2009;114(11):2323-32.
16. Porcheray F, Miklos DB, Floyd BH, Sarantopoulos S, Bellucci R, Soiffer RJ, et al. Combined CD4 T-cell and antibody response to human minor histocompatibility antigen DBY after allogeneic stem-cell transplantation. Transplantation. 2011;92 (3):359-65.
17. Zorn E, Miklos DB, Floyd BH, Mattes-Ritz A, Guo L, Soiffer RJ, et al. Minor histocompatibility antigen DBY elicits a coordinated B and T cell response after allogeneic stem cell transplantation. J Exp Med. 2004;199 (8):1133-42.
18. Griffioen M, van der Meijden ED, Slager EH, Honders MW, Rutten CE, van Luxemburg-Heijs SAP, et al. Identification of phosphatidylinositol 4-kinase type II beta as HLA class II-restricted target in graft versus leukemia reactivity. Proc Natl Acad Sci USA. 2008;105(10):3837-42.
19. Stumpf AN, van der Meijden ED, van Bergen CAM, Willemze R, Falkenburg JHF, Griffioen M. Identification of 4 new HLA-DR-restricted minor histocompatibility antigens as hematopoietic targets in antitumor immunity. Blood. 2009;114(17):3684-92.
20. Palmer I, Wingfield PT. Preparation and extraction of insoluble (inclusion-body) proteins from Escherichia coli. Curr Protoc Protein Sci. 2004;Chapter 6:Unit 6 3.
21. Schwenk JM, Lindberg J, Sundberg M, Uhlen M, Nilsson P. Determination of binding specificities in highly multiplexed bead-based assays for antibody proteomics. Mol Cell Proteomics. 2007;6(1):125-32.
22. Wardemann H, Nussenzweig MC. B-cell self-tolerance in humans. Adv Immunol. 2007;95:83-110.
23. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. Science. 2003;301(5638):1374-7.
24. Tsuiji M, Yurasov S, Velinzon K, Thomas S, Nussenzweig MC, Wardemann H. A checkpoint for autoreactivity in human IgM+ memory B cell development. J Exp Med. 2006;203(2):393-400.
25. Cambier JC, Gauld SB, Merrell KT, Vilen BJ. B-cell anergy: from transgenic models to naturally occurring anergic B cells? Nat Rev Immunol. 2007;7(8):633-43.
26. Kita H, Lian ZX, van de Water J, He XS, Matsumura S, Kaplan M, et al. Identification of HLA-A2-restricted CD8(+) cytotoxic T cell responses in primary biliary cirrhosis: T cell activation is augmented by immune complexes cross-presented by dendritic cells. J Exp Med. 2002;195(1):113-23.
27. Amigorena S. Fc gamma receptors and cross-presentation in dendritic cells. J Exp Med. 2002;195(1):F1-F3.
28. Dhodapkar KM, Krasovsky J, Williamson B, Dhodapkar MV. Antitumor monoclonal antibodies enhance cross-presentation ofcCellular antigens and the generation of myeloma-specific killer T cells by dendritic cells. J Exp Med. 2002;195(1):125-33.