Selective loss of vaccine-specific memory B cells in a rhesus macaque model of chemotherapy: influence of doxorubicin on immunological memory

Formation of a potent antibody (Ab) response following vaccination is considered to be a correlate of protective immunity. Chemotherapy can result in immunological memory loss by means of diminished antibody (Ab) titers, which is also observed in pediatric acute lymphoblastic leukemia (ALL) patients.[1] In line with this, earlier we found a link between diminished protective specific Ab titers and loss of bone marrow plasma cells (BMPCs) after completion of pediatric ALL therapy.[2] Some patients also fail to mount protective titers despite re-vaccination.[1] Duration and intensity of chemotherapy in relation to defective mounting and/or maintenance of immunological memory is unknown. Furthermore, it is not clear which cellular compartment(s) of the adaptive immunity are mostly affected and contribute to the defective titers, as formation of optimal B-cell responses commonly require T-cell help. B-cell memory is made up of both circulating antigen-specific Abs, produced by BMPCs, as well as antigen-specific memory B cells (MBCs) that can differentiate to Ab-secreting cells (ASCs) if a pathogen evades the pre-existing Ab repertoire.[3] Helper T-cell populations can be divided into naïve and memory, where the latter consists of two large subsets with distinct phenotype, migratory capacity and function.[4,5] Central memory T cells (T_{CM}) are believed to mainly re-circulate to lymph nodes and lack immediate effector functions, while effector memory T cells (T_{EM}) primarily home to peripheral sites and possess immediate effector functions.[3,4]

To address the effects of chemotherapy on different cellular components of immunological memory in a more comprehensive and systematic manner, we developed a novel rhesus macaque model and used the cytotoxic drug doxorubicin (Dx), which is widely included in human treatment protocols against a large number of neoplasms. Ten adult rhesus macaques, which were serologically verified as measles-immune but tetanus and rubella naïve, were included in the study. The animals were divided into two groups (n=5 per group) with matched weight and base-line anti-measles IgG titers (Figure 1). One group was treated with three increasing doses of Dx, whereas the other received sodium chloride under otherwise identical circumstances. All animals were immunized on study day 86 (for details see Figure 1). On day 73 all Dx-treated animals experienced severe bone marrow (BM) toxicity, where lower cell counts were confined to the white blood cell (WBC) population (*Online Supplementary Table S1*). Red blood cells were unaffected, and the platelet counts were even somewhat increased in the Dx-treated group (*data not shown*), which on the day of vaccination had reconstituted peripheral blood cell counts (*Online Supplementary Table S1*).

On study day 73, two weeks after the last Dx dose, the whole B-cell population, including $CD27^+$ MBCs and $CD27^{hi}CD38^{hi}$ plasma blasts, was almost entirely eradicated in the Dx treated group (Figure 2A and C-E). The recovery of total B-cell frequencies and MBCs varied greatly among Dx treated animals. B-cell frequencies were comparable to the control group from day 121 while MBC and plasma blast frequencies were comparable to controls from day 101 onward (Figure 2C-E). Likewise, measles-specific IgG^+ MBCs, quantified with ELISpot,[6] were eradicated after the last Dx dose in the treated animals, but had recovered already on day 101 (Figure 2F and G), which was similar to the pattern for peripheral IgG^+ MBCs (*Online Supplementary Table S1*). From day 101 onward, the mean measles-specific IgG+ MBC frequency was marginally lower in the Dx treated group, but the difference was not statistically significant (Figure 2G). Apart from one animal with marked increase of measles-specific MBC proportions, no significant booster effect on measles MBCs was observed (Figure 2G). If the rapid reconstitution of the MBC pool was due to preserved MBCs in other compartments, such as secondary lymphoid organs,[7] still has to be clarified.

It has previously been shown that several cytotoxic drugs induce more severe effects on B compared to T cells in peripheral blood,[8] and that memory T cells are preserved.[9]

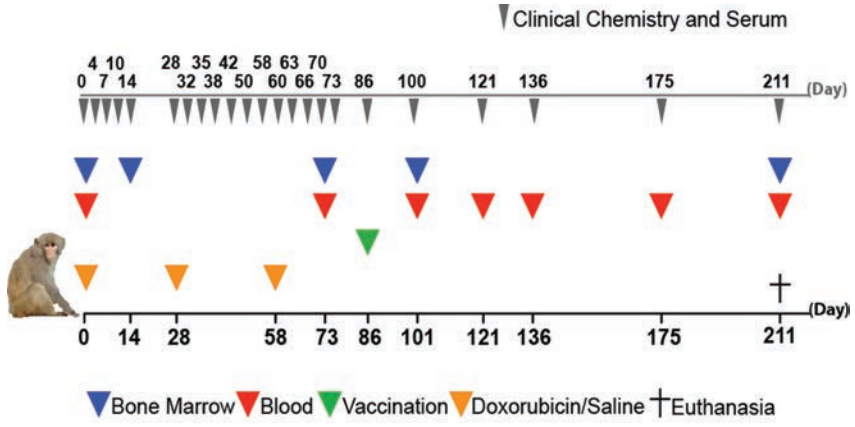

Figure 1. Ten measles-immune rhesus macaques, 11 to 15 years old, were included in the study. The chemotherapy group (n=5) was treated with 30 mg/m², 50 mg/m² and 75 mg/m² of doxorubicin diluted with 0.9% sodium chloride, infused for 60 min (orange triangles) and the control group (n=5) received saline (orange triangles) under identical conditions. Primary (rubella and tetanus) and secondary (measles) vaccine responses were evaluated after vaccination of all animals with 0.5 mL subcutaneous M.M.RVaxPro© (Sanofi Pasteur MSD) and 0.5 mL intramuscular Tetanus Toxoid Vaccine (Netherlands Vaccine Institute) on day 86. Blood (14 mL, red triangles) and bone marrow aspirate (blue triangles) were sampled for isolation of peripheral-blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs). For assessment of clinical chemistry, 1 mL blood (gray triangles on top) was taken and serum stored. Due to technical issues study day 0, there was not enough BM sample to perform all analyses in some animals. On the basis of unaltered neutrophil counts (*Online Supplementary Table S1*), 1 BM sample from the treated group and 3 BM samples from the control group which represent baseline values are from study day 14. At the end of the study the animals were euthanized by an overdose of pentobarbital. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of BPRC.

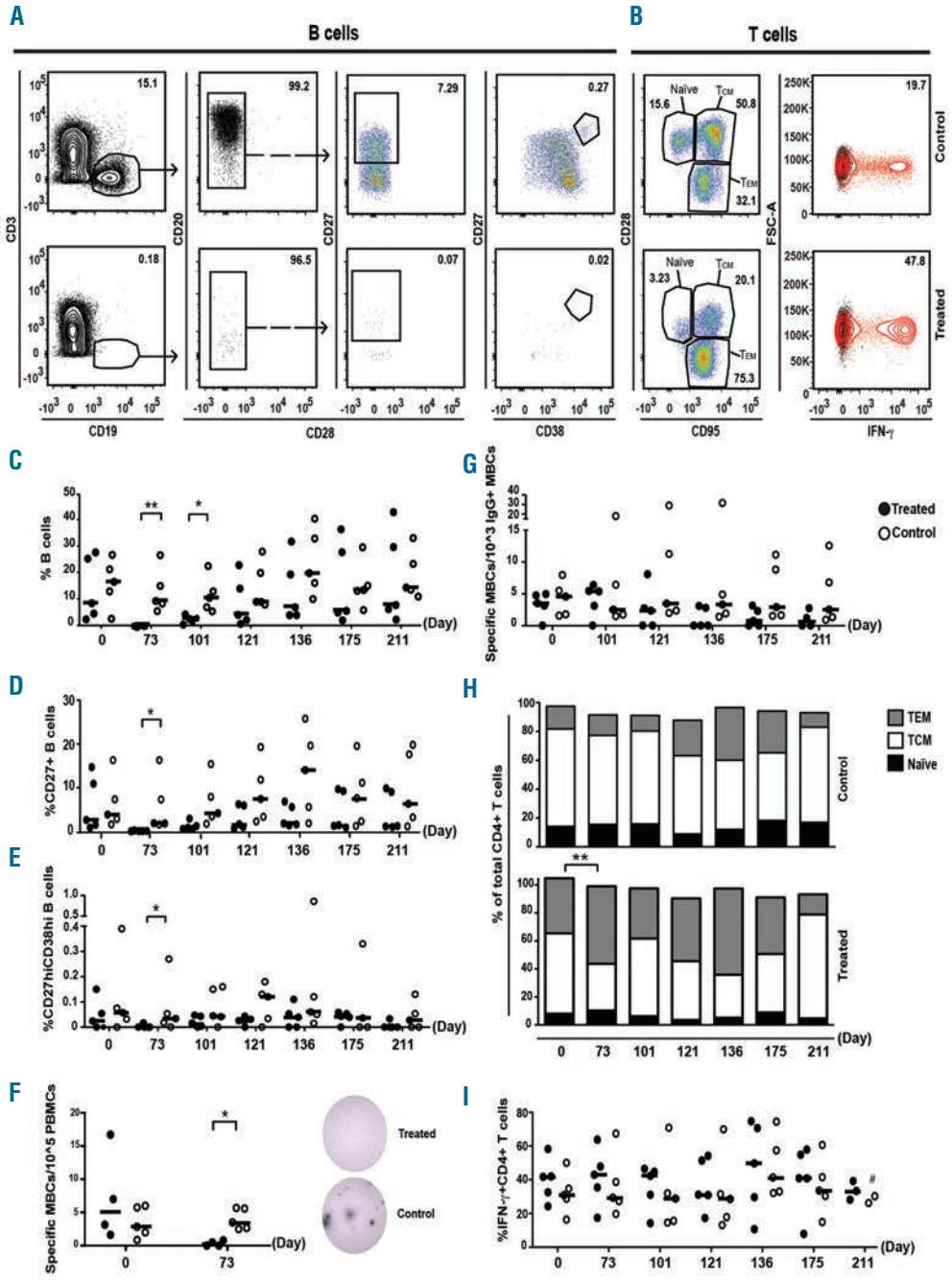

Figure 2. Assessment of B- and T-cell phenotype and function in peripheral blood (PB). For flow cytometric analysis, PB mononuclear cells were labeled with following pre-titrated fluorochrome-conjugated Ab combinations (if not otherwise stated from BD Biosciences): CD3 (SP34-2), CD4 (L200), CD19 (J3-119, Beckman Coulter), CD20 (L27), CD27 (MT271), CD28 (CD28.2), CD38 (AT.1, StemCell), CD95 (DX2), CD138 (DL-101), and IFN-γ (5 mg/mL B27). Further, a Live/Dead© probe (Life Technologies) was used. For detection of intracellular T cell IFN-γ, PBMCs were activated with 5 ng/mL Phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL Ionomycin (both from Sigma-Aldrich) in the presence of protein transport-inhibitor monensin (BD Biosciences). Data were acquired with BD LSR II and analyzed with FlowJo (v.10, TreeStar Inc.). (A) Representative B-cell and (B) T-cell gating on day 73 where B cells were defined as viable $CD3^-$, $CD19^+$, CD28-, $CD20^{+/-}$ single cells; memory B cells (MBCs) as $CD27^+$ B cells and circulating plasma blasts as $CD27^{hi}$, $CD38^{hi}$ B cells. Helper T cells were defined as viable $CD4^+$, $CD19^-$, $CD20^-$, $CD3^+$ single cells in the lymphocyte gate. Naïve T cells were defined as $CD28^+$, CD95- helper T cells; central memory (TCM) T cells as $CD28^+$, $CD95^+$ helper T cells and effector memory (TEM) T cells as $CD28^-$, $CD95^+$ helper T cells. (C) Percentage of B cells of gated live single cells was significantly lower in Dx treated group on day 73 (P=0.008) and day 101 (*P*=0.030). On day 73 Dx treated animals had lower (D) percentage of MBCs (*P*=0.012) and (E) plasmablasts (*P*=0.045) of gated live single cells compared to controls. (F) Representative ELISPOT wells and collected data of measles-specific MBCs/10^5 PBMCs day 0 and 73 (*P*=0.011, day 73). (G) Measles-specific MBCs/10^3 IgG^+ MBCs between day 0 and 211, where ratios between days 101-211 were somewhat lower in the treated group (ANOVA, *P*=0.053). (B and H) Composition of naïve and memory helper T cells where bars represent median values of naïve T-cell proportions (black), TCM proportions (white) and TEM proportions (gray). The frequencies of TCM were significantly reduced in Dx group between day 0 and 73 compared to the control group (*P*=0.008). (B and I) Percengate IFN-γ^+ cells among $CD4^+$ T cells [displayed as red overlays in (B) and collected data in (I) based on values after subtraction from donor-matched non-stimulated cultures]. Horizontal bars represent median values, (open circles) Control, (filled circles) Dx. #Median not applicable.

In line with this, neither CD3+ lymphocyte proportions, helper T-cell proportions (data not shown), the proportion of total memory T cells in peripheral blood (Figure 2B and H) or T-cell functional capacity (Figure 2I) was affected throughout the study period. However, chemotherapy was associated with altered proportions of peripheral helper T-cell subsets with higher proportions of mainly TEM cells (Figure 2H). Contrary to CD28+ T cells, CD28- T cells have been associated with a lower proliferative potential.[10] Therefore, one likely reason behind increased peripheral TEM cell proportions could be that they are relatively more resistant to Dx intercalation and thereby enriched in Dx-treated animals. Another possible and not mutually exclusive explanation could be that TCM cells differentiate further into TEM cells due to increased immune surveillance after immunosuppression by Dx. However, the significance of the observed subset redistribution in relation to our functional readout is unclear as helper T-cell IFN-γ production capacity *in vitro* was similar between the groups (Figure 2I). Furthermore, all animals had an increase of TEM cell proportions among helper T cells after immunization (analysis of variation over time measured by ANOVA, P=0.001).

Contrary to the peripheral MBC compartment, measles-specific IgG titers in serum were largely unaffected following chemotherapy, between day 0 and 73 (Figure 3A). A small booster effect on the anti-measles titers was noticed following vaccination (day 86) and day 121 in both groups. As the half-life of IgG in macaques is approximately eight days,[11] the Ab production was most likely continuous in the treated animals. In support of this, neither the BM proportions of total PCs (Figure 3B), total IgG+ PCs (Figure 3C) or measles-specific IgG+ PCs proportions (Figure 3D) were markedly affected between days 0 and 73. Similar to observations in peripheral blood (Figure 2), total BM CD19+ cell proportions (as determined by flow cytometry) (*Online Supplementary Figure 2A*) were diminished following chemotherapy but recovered with time (*Online Supplementary Figure 2B*). Interestingly, our observation based on individual animals suggested that within the CD19+ BM-cell population the CD38hiCD138+ fraction, which corresponds to PCs, was rather unaffected by Dx treatment since PC frequencies were stable or even increased from day 0 to 73 in Dx-treated animals as compared with controls (*Online Supplementary Figure 2C*).

Due to limited data, it is unclear whether immunization is feasible during or closely after chemotherapy. This uncertainty may impact quality of life in patients and be a health threat if new infectious epidemics occur. Current recommendations span from three to 12 months after completion of therapy.[12] To address the question as to whether *de novo* vaccination is feasible during B-cell recovery phase, animals were immunized against rubella and tetanus one month after the last Dx dose. Overall, both groups were equally potent in mounting specific IgG titers following vaccination, although the Dx-treated animals responded slower initially (Figure 4A and B). Likewise, rubella-specific MBC frequencies were also significantly lower in the treated group initially, and their subsequent enrichment over time was somewhat smaller (Figure 4C). Tetanus-specific MBCs also expanded slower in the treated group initially, but later on to the same extent in both groups (Figure 4D). A smaller size of responding B-cell clones upon immunization might be one reason behind altered expansion and kinetics in Dx-treated animals compared to controls. Yet Dx-treated animals had a relatively preserved ability to form MBCs against *de novo* antigens that might be explained by a GC response sustained long enough to allow B-cell recovery. In mice, it has been demonstrated that functional GCs can be sustained for up to eight months after vaccination.[13]

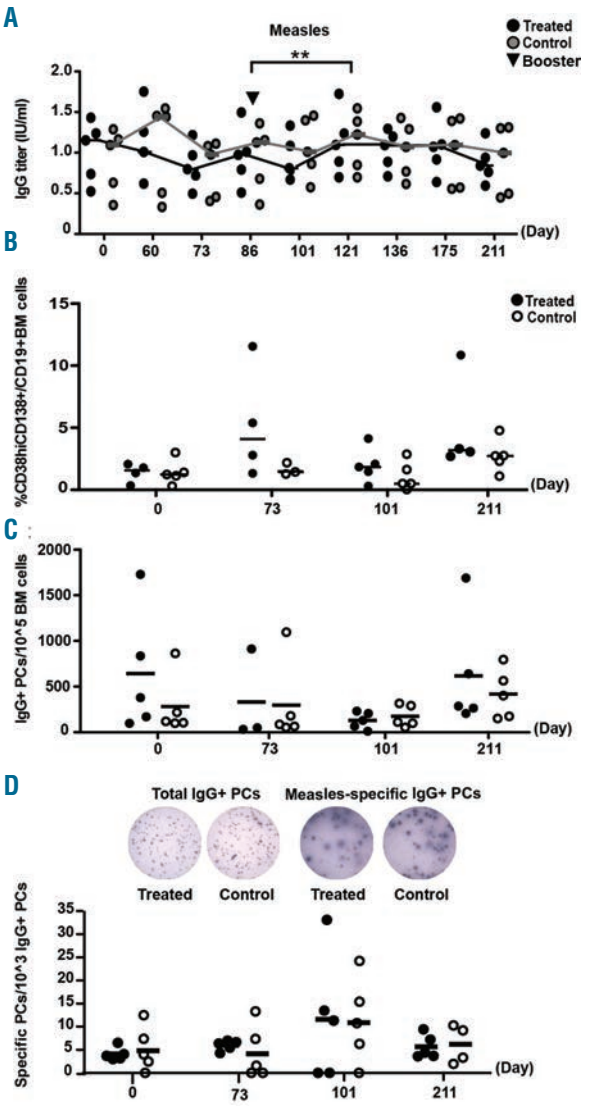

Figure 3. Evaluation of doxorubicin effect on earlier-acquired protective titers and secondary B-cell responses against measles. (A) Serum anti-measles IgG titers were determined with Enzygnost® ELISA (Siemens, P=0.002 between day 86 and 121 for all animals jointly). The relative Ab titer increase did not differ between the groups (P=1.0). (B) BMPCs were assessed with flow cytometry and defined as viable CD19+, CD20+/-, CD38hi, CD138+ single cells, shown as percentage of CD19+ BM cells. Proportions of (C) total IgG+ and (D) measles-specific IgG+ BMPC determined by ELISPOT. Representative wells from day 73 are shown. No statistically significant changes were noticed over time.

We asked if the robust IgG response to vaccinations observed in Dx treated animals during B-cell recovery phase also could convert to long-lived memory. This conversion occurs when Ab production shifts from peripheral plasma blasts to long-lived BMPCs and appears variable and dependent on type of antigen.[14] In our study, we noticed that rubella-specific BMPCs formed equally well in both groups (Figure 4E), and both rubella and measles IgG titers correlated strongly to the corresponding specific BMPCs already four months after vaccination (measles: Spearman r=0.80, P=0.014; rubella: r=0.90, P=0.0046), which implies a conversion to long-term immunity. The correlation was equally strong in both Dx treated and control animals,

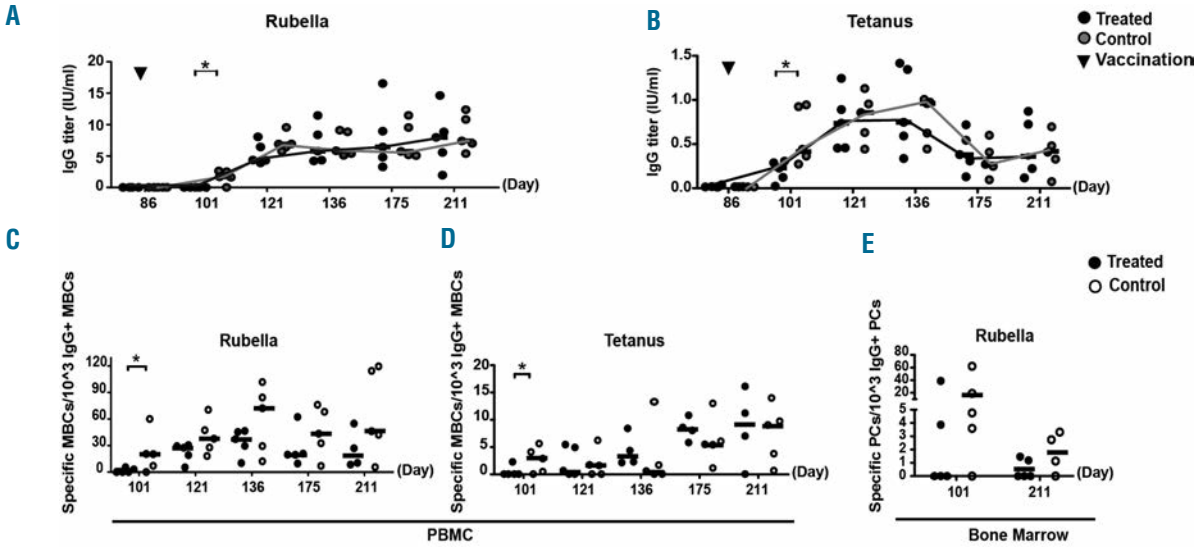

Figure 4. Evaluation of B-cell responses to *de novo* rubella and tetanus toxoid vaccination. (A and B) IgG titers were determined with Enzygnost® ELISA. (C and D) Antigen-specific MBCs and (E) BMPCs were quantified with ELISPOT. Proportions of (C) rubella-specific and (D) tetanus-specific MBCs. Specific IgG titers were significantly lower in the treated group on day 101 against both rubella (A) (*P*=0.025) and tetanus (B) (*P*=0.032). On day 101, the number of specific MBCs was significantly lower in the treated group for rubella (C) (*P*=0.002) and tetanus (D) (*P*=0.05). The mean rubella-specific MBC ratio increase from day 101 to 211 was lower in the treated group, but this difference was not statistically significant (*P*=0.083). (E) Rubella-specific BMPC proportions with no significant differences between the groups.

demonstrating a preserved ability to mount a long-term vaccine response during B-cell recovery phase after chemotherapy. If these findings translate into cancer patients, vaccine responses post chemotherapy should be followed-up and assessed for a longer period of time. It should be emphasized, however, that our non-human primate study here does not take into account that previous chemotherapy treatment(s) in humans or disease *per se* possess suppressive effects on the adaptive immune system.

To summarize, circulating B cells are more sensitive to Dx treatment than T cells and BMPCs. Here, immunization shortly after chemotherapy resulted in successful mounting and persistence of IgG responses to vaccines despite a constrained peripheral B-cell population. In contrast to the prolonged suppression of the BM in cancer patients, our finding of a stable BMPC pool indicates that short-term chemotherapy is not enough to induce PC depletion and subsequent loss of specific IgG titers, implying that patients who loose protective Abs likely have a diminished BMPC pool.

Hanna M. Ingelman-Sundberg,[1] Shanie Saghafian-Hedengren,[1] Maja Jahnmatz,[2] Staffan Eksborg,[1] Margreet Jonker,[3] and Anna Nilsson[1]

[1]Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden; [2]Public Health Agency of Sweden, Solna, Sweden; and [3]Biomedical Primate Research Center, Rijswijk, The Netherlands

Funding: this work was supported by grants from PRIMOCID, (EUPRIM-NET, contract number 026155), the Stockholm County through the ALF agreement and Karolinska Institutet central funding.

Correspondence: Anna.Nilsson.1@ki.se

doi:10.3324/haematol.2014.116111

Key words: doxorubicin, B cells, T cells, BMPC, response to vaccines, chemotherapy.

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. van Tilburg CM, Sanders EA, Rovers MM, et al. Loss of antibodies and response to (re-)vaccination in children after treatment for acute lymphocytic leukemia: a systematic review. Leukemia. 2006;20(10): 1717-1722.
2. Nilsson A, De Milito A, Engstrom P, et al. Current chemotherapy protocols for childhood acute lymphoblastic leukemia induce loss of humoral immunity to viral vaccination antigens. Pediatrics. 2002;109(6):e91.
3. Amanna IJ, Slifka MK. Contributions of humoral and cellular immunity to vaccine-induced protection in humans. Virology. 2011; 411(2):206-215.
4. Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. Nat Rev Immunol. 2014;14(1):24-35.
5. Pitcher CJ, Hagen SI, Walker JM, et al. Development and homeostasis of T cell memory in rhesus macaque. J Immunol. 2002;168(1):29-43.
6. Jahnmatz M, Kesa G, Netterlid E, Buisman AM, Thorstensson R, Ahlborg N. Optimization of a human IgG B-cell ELISpot assay for the analysis of vaccine-induced B-cell responses. J Immunol Methods. 2013.
7. Yoshida T, Mei H, Dorner T, et al. Memory B and memory plasma cells. Immunol Rev. 2010;237(1):117-139.
8. van Wering ER, van der Linden-Schrever BE, Szczepanski T, et al. Regenerating normal B-cell precursors during and after treatment of acute lymphoblastic leukaemia: implications for monitoring of minimal residual disease. Br J Haematol. 2000;110(1):139-146.
9. Haining WN, Neuberg DS, Keczkemethy HL, et al. Antigen-specific T-cell memory is preserved in children treated for acute lymphoblastic leukemia. Blood. 2005;106(5):1749-1754.
10. Akbar AN, Fletcher JM. Memory T cell homeostasis and senescence during aging. Curr Opin Immunol. 2005;17(5):480-485.
11. Challacombe SJ, Russell MW. Estimation of the intravascular half-lives of normal rhesus monkey IgG, IgA and IgM. Immunology. 1979; 36(2):331-338.
12. Rubin LG, Levin MJ, Ljungman P, et al. 2013 IDSA clinical practice guideline for vaccination of the immunocompromised host. Clin Infect Dis. 2014;58(3):e44-100.
13. Dogan I, Bertocci B, Vilmont V, et al. Multiple layers of B cell memory with different effector functions. Nat Immunol. 2009;10(12):1292-1299.
14. Amanna IJ, Slifka MK. Mechanisms that determine plasma cell lifespan and the duration of humoral immunity. Immunol Rev. 2010;236: 125-138.