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Quantitative tracking of T cell clones after hematopoietic stem cell transplantation

Ilgar Z. Mamedov, Olga V. Britanova, Dmitriy Bolotin, Anna V. Chkalina, Dmitriy B. Staroverov, Ivan V. Zvyagin, A. A. Kotlobay, M. A. Turchaninova, Denis A. Fedorenko, Andrew A. Novik, George V. Sharonov, Sergey Lukyanov, Dmitriy M. Chudakov and Yuriy Lebedev

Corresponding author: Dmitriy Chudakov, Institute of Bioorganic Chemistry, RAS

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23 December 2010

Thank you for the submission of your manuscript "Quantitative tracking of T cell clones after hematopoietic stem cell transplantation" to EMBO Molecular Medicine.

As you have seen, the reviewers find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

In particular, reviewer #2 feels that the study would be strengthened by the separation of CD4+ and CD8+ T cells in the analysis. Importantly, reviewer #1 feels that the potentially valuable software should be publicly available. Of note, both reviewers #1 and #2 feel that specific statements regarding the long-term reconstitution of the T cell repertoire or the treatment regimen, respectively, should be moderated.

To clarify again, we would consider the manuscript as a short Report. Please refer to our Instructions to Authors regarding the format (http://embomolmed.msubmit.net/html/Author_Instructions.pdf). Nevertheless, this should leave you enough space to transfer some supporting text and figures (for example regarding the amplification technique and web client) into the main text to increase the impact of your study on the broad audience EMBO Molecular Medicine aims to reach.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the space and time constraints

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outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Reviewer #1 (Remarks to the Author):

This study describes a new methodological approach to describing TCR repertoires that takes advantage of recent advances in high-throughput sequencing technology. The method is applied to obtain proof-of-principle that the method is suitable to analyze the clonal composition of peripheral blood in a single patient who underwent haematopoietic stem cell transplantation.

The laboratory and bioinformatics work is well done and the paper is of interest mostly as a technological advance with great potential to support significant advancements in immunology and haematology. However there are two major concerns.

(1) This is clearly a proof of principle study and it should be described as such. The follow-up duration is limited to 10 months post-transplant. Therefore statements such as:
- in the Abstract: "...the first detailed, quantitative, long-term tracking of T cell clones after transplantation."

- and on page 3 "This arsenal of tools was employed to perform the first long-term, deep, and quantitative tracking of the fate of T cell clones..." and "2. HSCT decreased overall diversity of T cell clones (Supplementary Fig. 6), while the number of "hyper-expanded" clones (comprising >1% of all TCR beta sequences) increased, leading to the propagation of specific TCR V beta gene families (Supplementary Fig. 7)." .

are overstatements and the proposed interpretations are misleading in that they generalize to long-term follow-up observations that are shown only for relatively early follow-up (<1year).

- and page 4. Clones which survived established a new balance that was unexpectedly stable, as revealed by in silico analysis, demonstrating that the spectratypes and clonotypes skewed by HSCT remained intact over the next 6 months (Fig. 2). Restoration of a full TCR beta repertoire which would re-produce the previous diversity and the clonal expansions normally present in healthy subjects, would probably take years, especially given the low abundance of naive T cells observed after HSCT (Supplementary Fig. 8)."

Muraro et al 2005 have already shown that recovery and rebound of thymic output requires 2 years post-transplantation and that the early stages (first year) post transplantation is dominated by limited diversity and oligoclonal expansions. As the Authors only analysed one subject at pre-transplant and at 6 and 10 months post-transplant, they should not draw any strong conclusions about long-term reconstitution of immune repertoire.

Therefore, the Authors should either provide longer term analysis (2 years of follow-up or longer), or greatly temper their interpretations and conclusions in order to reflect that they have only examined immune reconstitution during, and not even up to, the first year post-transplantation.

(2). TRbase software. The software is a central piece of this report, and seems a very valuable tool. However, a major concern is lack of information in the manuscript about public availability of the

software. The Authors should indicate whether, when and under which conditions they intend to make their software publicly available and declare their commitment on how they intend to comply with these policies. Ideally the software should be web-based (they already show a web client) and free of charge for academic institutions. Of course the Authors are free to pursue commercializing the software but in that case they should not expect to also be able to advertise it through a publication.

In addition, the software should be renamed since the denomination is already being used by a publicly available database (TRbase: A Database Of Tandem Repeats In The Human Genome. Maintained by the School of Biosciences, University of Exeter, UK.).

Minor points:

(1). Introduction "The results of most studies indicate complete or nearly complete T cell repertoire "renovation" and depletion of autoreactive clones2-4, although some studies have indicated that pre-existing T cells can survive the conditioning regimen 5.".

The Authors should quote more accurately the findings by Dubinsky et al (ref. 4 in their manuscript) where in fact the article focused on describing the persistence of clonal specificities post-transplantation and on exploring the origin of the persisting clones.
(2). It would be helpful to report, in addition to the starting amount of RNA needed for analysis, the number of cells needed.

Reviewer #2 (Remarks to the Author):

In this paper Mamedov et al., present an optimized technique for high throughput analysis of TCR repertoires. The technique is then used to track T cell clones following autologous hematopoietic stem cell transplantation for refractory ankylosing spondylitis. The paper is well written and the results are clearly presented.

The described optimized TCR beta amplification technique seems solid and is well presented. Due to limited in dept knowledge about the technical part this review report will focus on the application of the technique.

Autologous hematopoietic stem cell transplantation (aHSCT) is used as a treatment for refractory autoimmune disease. This therapy has proven effective but the underlying mechanism remains to be elucidated. One of the primary rationales for using aHSCT therapy has been the hypothesis that intense immune ablation followed by stem cell transplantation can wipe out an autoaggressive immune system and promoting the regeneration of a healthy immune system. Later it has been shown that T cells may survive the conditioning and this has fuelled the debate about the intensity of the myeloablative conditioning regimens (limited by toxicities). The optimized TCR beta amplification technique described in this manuscript makes extensive quantitative tracking of T cell clones following HSCT possible, using only small samples.

The authors present quantitative tracking of the fate of T cell clones in a patients with ankylosing spondylitis before and after aHSCT. They show that a substantial number of T cell clones survive the conditioning regimen and also expand after aHSCT.

1. The mobilized stem cells used for transplantation were not selected for CD34+ and therefore likely contained T cells. In vivo T cell depletion was performed by ATG infusion but it is not clear for how long and on what days (supplementary methods). The authors should add this information and can the authors comment on the possible re-infusion of T cell clones with the autograft.

2. The authors comment that a large number of TCR beta CD3 amino acid sequences from the patient are 100% identical to CD3 variants already referenced in the NCBI protein database. They conclude that despite the huge potential diversity of the T cell receptors in humans, the TCR sequence pool can be rather restricted. This is an interesting observation. In line with this, how sure are the authors that "persistent" T clones measured after aHSCT have not been de novo generated? It

is generally assumed that de novo generation of identical TCR sequences is rare but their observation about clonal restriction may suggest otherwise.

3. All the sequencing work has been done on total PBMC. The study would gain strength by analyzing both the CD4+ and CD8+ T cell repertoires.

4. The authors conclude that chemotherapy regimens for the treatment of autoimmune diseases should be moderated to preserve the clonal diversity of immune cells. However, to draw this conclusion they should compare different conditioning regimens and analyze the clonal diversity following these treatments.

Reviewer #3 (Remarks to the Author):

The paper by Mamedov et al. reports the development of experimental and bioinformatic approaches and their combined use to monitor the fate of T cell clones in a patient with ankylosing spondylitis subjected to hemopoietic stem cell transplantation.

The methods are sound and demonstrated to provide an unbiased quantitative assessment of the T cell repertoire through massive sequencing of RT-PCR-amplified Vbeta genes, revealing novel features such as the survival and re-expansion of functional T cell clones. The two Figures included while informative are poorly presented and would be unreadable in the condensed format.

The short report is rather technical and descriptive, and does not provide an advance in understanding the mechanisms generating or regulating the T cell repertoire in the perturbed conditions represented by the auto-immune disease studied or the transplanation scheme used to treat it. It therefore would be suitable for a journal specialized in functional genomics methods and/or their application to the field of immulology/transplantation.

1st Revision - authors' response 07 January 2011

We wish to thank both reviewers for the thorough work with our manuscript.
We did our best to clarify all the issues: please see point by point response below.

Reviewer #1 (Remarks to the Author):

(1) This is clearly a proof of principle study and it should be described as such. The follow-up duration is limited to 10 months post-transplant. Therefore statements such as: - in the Abstract: "...the first detailed, quantitative, long-term tracking of T cell clones after transplantation." are overstatements and the proposed interpretations are misleading in that they generalize to long-term follow-up observations that are shown only for relatively early follow-up (<1year).

We changed to
“Here we describe an optimized RNA-based technology for unbiased amplification of T cell receptor beta-chain libraries and use it to perform the first detailed, quantitative tracking of T cell clones during 10 months after transplantation.”

- and on page 3 "This arsenal of tools was employed to perform the first long-term, deep, and quantitative tracking of the fate of T cell clones..."

We changed to:
“first deep, quantitative tracking”

"2. HSCT decreased overall diversity of T cell clones (Supplementary Fig. 6), while the number of "hyper-expanded" clones (comprising >1% of all TCR beta sequences) increased, leading to the propagation of specific TCR V beta gene families (Supplementary Fig. 7)."

We just describe the results – we see no overstatement here.

- and page 4. Clones which survived established a new balance that was unexpectedly stable, as revealed by in silico analysis, demonstrating that the spectratypes and clonotypes skewed by HSCT remained intact over the next 6 months (Fig. 2). Restoration of a full TCR beta repertoire which would re-produce the previous diversity and the clonal expansions normally present in healthy subjects, would probably take years, especially given the low abundance of naive T cells observed after HSCT (Supplementary Fig. 8)." Muraro et al 2005 have already shown that recovery and rebound of thymic output requires 2 years post-transplantation and that the early stages (first year) post transplantation is dominated by limited diversity and oligoclonal expansions.

Yes, but here we directly demonstrate that these expansions are the clones that survived transplantation, and that these expansions generally remain the same between the 4 and 10 months after HSCT.

We added note "**in the patient blood**" to this paragraph (page 4, paragraph 5), just to indicate that we only describe the results for this particular patient.

As the Authors only analysed one subject at pre-transplant and at 6 and 10 months post-transplant, they should not draw any strong conclusions about long-term reconstitution of immune repertoire. Therefore, the Authors should either provide longer term analysis (2 years of follow-up or longer), or greatly temper their interpretations and conclusions in order to reflect that they have only examined immune reconstitution during, and not even up to, the first year post-transplantation.

We excluded the term "long-term". All our conclusions and interpretations are limited to the 10-month period.

(2). TRbase software. The software is a central piece of this report, and seems a very valuable tool. However, a major concern is lack of information in the manuscript about public availability of the software.

We hope that the software algorithms are described in sufficient detail in the supplementary part, so the ideas proposed and realized can be implied in further works. As it is, the software is necessarily linked to our internal server database and infrastructure, and can not work effectively as an open source.

The general idea of not just to analyze and view, but to perform cross-identity and cross-blast analysis of multiple datasets, makes it challenging to realize as an open source. We are currently working on the next version of the software based on the similar algorithms, that we hope will be more flexible. However, this will take at least several months.

In general, we believe that international open web resource should be developed, linked to the classified database of TCR V beta CDR3 regions, based on a strong international resource, such as NCBI or IMGT. Although we will be definitely happy to contribute to the development of such resource and database, it is technically out of our power to generate and support it ourselves.

In addition, the software should be renamed since the denomination is already being used by a publicly available database (TRbase: A Database Of Tandem Repeats In The Human Genome. Maintained by the School of Biosciences, University of Exeter, UK.).

We renamed the software to the "TCRbase".

Minor points:

(1). Introduction "The results of most studies indicate complete or nearly complete T cell repertoire

"renovation" and depletion of autoreactive clones 2-4, although some studies have indicated that pre-existing T cells can survive the conditioning regimen 5.". The Authors should quote more accurately the findings by Dubinsky et al (ref. 4 in their manuscript) where in fact the article focused on describing the persistence of clonal specificities post-transplantation and on exploring the origin of the persisting clones.

Yes, we apologize for this inaccuracy. We removed this citation of Dubinsky et al., since it is not appropriate in this context.

This reference is now cited below, in the context of discussion of CD34+ selected grafts.

(2). It would be helpful to report, in addition to the starting amount of RNA needed for analysis, the number of cells needed.

As indicated, the starting RNA amount was "**2 µg of total RNA obtained from PBLs purified from a 2-ml blood sample**".
To make it more clear, we added the approximation (page 3, paragraph 4):
"**containing approximately 2 million of T cells**"

Reviewer #2 (Remarks to the Author):

1. The mobilized stem cells used for transplantation were not selected for CD34+ and therefore likely contained T cells. In vivo T cell depletion was performed by ATG infusion but it is not clear for how long and on what days (supplementary methods). The authors should add this information and can the authors comment on the possible re-infusion of T cell clones with the autograft.

We have added following paragraph to discuss this point (page 5, paragraph 4):

"The evolution of HSCT therapy protocols will require further studies of TCR repertoires before and after HSCT in order to understand the fate and the role of T cell clones in more detail. In particular, the fates of various subpopulations of T cells are of significant interest, including those of regulatory T cells and Th17 cells. Besides, it is important to study dependence of changes in the T cell repertoire on different graft manipulation protocols, such as $CD34^+$-positive selection or negative purging of lymphocytes subsets by monoclonal antibodies {Farge, #12;Moore, 2002 #10}. While it was shown that CD34+ selected grafts do not contain large amounts of T-cell clones observed after HSCT {Dubinsky, 2010 #8}, $CD34^+$-positive selection was not performed in our case, and thus re-infusion of T cell clones with the autograft can not be excluded."

2. The authors comment that a large number of TCR beta CD3 amino acid sequences from the patient are 100% identical to CD3 variants already referenced in the NCBI protein database. They conclude that despite the huge potential diversity of the T cell receptors in humans, the TCR sequence pool can be rather restricted. This is an interesting observation. In line with this, how sure are the authors that "persistent" T clones measured after HSCT have not been de novo generated? It is generally assumed that de novo generation of identical TCR sequences is rare but their observation about clonal restriction may suggest otherwise.

The observed clonal restriction is characteristic for amino acid CDR3 sequences, while tracking of clonal sequences was, of course, performed for the nucleotide ones. Although we can not be absolutely sure about each particular clone, the general probability of "de novo" generation of identical nucleotide CDR3 variants was considered as negligible.

3. All the sequencing work has been done on total PBMC. The study would gain strength by analyzing both the CD4+ and CD8+ T cell repertoires.

Indeed, it would be of interest, as well as separate sequencing of Th17 and Treg populations. In our next works we will definitely consider this option. However, here we tried to perform it all as

quantitative as possible. This means - from blood to RNA with minimal intermediate steps. Therefore, we avoided separation by FACS or magnetic beads.

4. The authors conclude that chemotherapy regimens for the treatment of autoimmune diseases should be moderated to preserve the clonal diversity of immune cells. However, to draw this conclusion they should compare different conditioning regimens and analyze the clonal diversity following these treatments.

Of course, we do not appeal to change the protocols according to our single patient-based studies. We only indicate that “our data support the concept that chemotherapy regimens for the treatment of autoimmune diseases should be significantly moderated”.

Reviewer #3 (Remarks to the Author):

The two Figures included while informative are poorly presented and would be unreadable in the condensed format.

The current figures are pdf versions that are easy to include in the *.doc file. Original figures are in *.eps format. Thus all details, including font sizes, can be changed as required.

2nd Editorial Decision 02 February 2011

Thank you for the submission of your revised manuscript "Quantitative tracking of T cell clones after hematopoietic stem cell transplantation" to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. I would like you to incorporate the changes detailed below before we can proceed with the official acceptance of your manuscript.

As you will see, the reviewers acknowledge that the manuscript was significantly improved during revision. However, while reviewer #2 indicates that the manuscript is suitable for publication, reviewer #1 raises an issue that should be addressed. We appreciate that you describe in detail the algorithms used in the software. We also agree with the reviewer that the TCRbase software is central to the present study. For publication of your study, it would thus be ideal, but not absolutely necessary, to release the source code. However, it is mandatory to include a statement in Material and Methods that non-commercial and academic users are granted free access to the TCRbase implementation described in this paper.

Attached, please also find the edited article file as well as the supplementary data file. Please go carefully through the introduced changes, which are highlighted, to ensure that no intended meaning was changed inadvertently and accept or decline them.

Revised manuscripts should be submitted within two weeks of a request for revision; they will otherwise be treated as new submissions, unless arranged differently with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Remarks to the Author):

The Authors have addressed most points and the manuscript has improved.

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It is understandable that the Authors' group may not have the resources to run the software as a publicly available internet-based platform. However, to meet the published journal's policies, quoted in my review to the original submission, a statement that the source code will be made available at no charge to academic researchers must be added.

On a more minor note, grammar and language precision need editing such as on page 3 "2 million of cells" should be corrected and on page 4 "in the patient blood" (should be corrected as "in the patient's blood").

Referee #2 (Remarks to the Author):

suitable for publication

2nd Revision - authors' response 09 February 2011

Thank you so much for the thorough work with our manuscript entitled
"**Quantitative tracking of T cell clones after hematopoietic stem cell transplantation**".

We have performed the required changes, including:

1. We have released the source code and added the link within the supplement, part **Development of software for deep analysis**:
"Non-commercial and academic users are granted free access to the TCRbase implementation described in this paper. Source code is available at no charge:
http://sourceforge.net/projects/tcrbase/files/v0.1/ . "

2. In **Materials and Methods**, we have added necessary information (page 6, paragraph 3):
"The study was approved by local ethical committee. Patient gave written informed consent prior to enrolling in the study."

3. We are submitting the raw sequence data to the NCBI SRA database. The accession number was added to the manuscript (page 4, 1st paragraph):
"**raw data are available at NCBI SRA database, study accession number SRP005664**"

4. We have introduced all the proposed corrections to the manuscript and supplementary files. The only minor change is on page 5, paragraph 2:
"the TCR sequence pool recognizing key antigenic peptides **can** appear to be rather restricted, at least in subjects sharing the same Class I MHC alleles."

5. If possible, we wish to add one more author, Kotlobay A.A. I apologize for this late addition, but this work has long history and many people were involved at various stages.

We hope that the manuscript is now acceptable for publication.

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