**The effect on lymphocyte subsets of decreasing/stopping tyrosine kinase inhibitor therapy in chronic myeloid leukaemia: data from the DESTINY trial.**

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 Several studies have examined stopping TKI in chronic phase chronic myeloid leukaemia (CML) in stable MR4 (*BCR-ABL1/ABL1* transcript ratio < 0.01%). Recent data suggest that about 40% of such patients will lose major molecular response (MMR; *BCR-ABL1/ABL1* transcript ratio rising above 0.1%) on TKI discontinuation (reviewed in Saussele et al 2016 and Hughes and Ross 2016). Little is known about immunological mechanisms of continued leukaemic restraint after TKI withdrawal. All TKIs have potential immunosuppressive effects (reviewed in Steegmann et al 2012), although the clinical relevance of this is uncertain.

 Here we report on trends in immunological subsets in patients at our site entering the clinical trial DESTINY (De-Escalation and Stopping Treatment of Imatinib, Nilotinib or sprYcel), which has a unique design of initial TKI de-escalation for 12 months (to half the standard dose - imatinib 200mg daily, dasatinib 50mg daily or nilotinib 200mg twice daily), followed by complete cessation. Unlike other TKI stopping trials, entry was open to patients in stable MMR but not MR4, as well as the conventional group of patients in stable MR4. The detailed protocol has been previously described (Clark et al 2017). Key entry criteria were first chronic phase of CML, receiving either the same TKI for at least 3 years since diagnosis or had switched only once for initial drug intolerance, and all *BCR-ABL1/ABL1* transcript ratios in the 12 months before trial entry ≤ 0.1%. Molecular monitoring was carried out centrally (by author LF) monthly. Molecular recurrence was defined as loss of MMR.

 Lymphocyte subsets were examined by flow cytometry at trial entry, after 6 and 12 months of half-dose therapy, and after a subsequent 6 months of complete cessation, in all 39 DESTINY trial entrants from our site at the Royal Liverpool University Hospital; their details are given in Supplementary Table 1. The subsets studied were naïve (CD45RA+ CCR7+ CD27+), central memory (CD45RA- CCR7+) and effector memory (CD45RA- CCR7-) T cells, in each case in both CD4+ and CD8+ compartments, together with CD4+ regulatory (CD25+/CD127neg/low) T-cells and CD56dim and CD56bright NK cells.

 Levels of CD4+ regulatory T cells, CD56dim and CD56bright NK cell subsets were significantly lower at 12 months than at baseline (Figure 1, panels A-C; all p < 0.001); the CD56dim subset was also significantly lower at 6 months compared with baseline (p = 0.0032). No significant changes were seen in any other subset during the de-escalation phase. Between 12 and 18 months (i.e. the subsequent stopping phase), no changes were seen in any subset. No changes were seen in absolute lymphocyte numbers over either the de-escalation or the stopping phases, either in the entire study population or when stratified according to molecular recurrence.

 Logistic regression was used to explore the relationship between temporal changes in lymphocyte subsets and molecular recurrence (seen in 18 patients; 3 during de-escalation and 15 during the stopping phase). No significant association was seen between any lymphocyte subset changes in the first 6 months and molecular relapse. However, a rise in effector memory CD8+ cells between 6 and 12 months was associated with an increased risk of molecular recurrence (odds ratio [OR] = 1.86; 95% confidence interval [CI] = 1.06-3.64; p = 0.03); a similar effect was seen for this subset between baseline and 12 months (OR = 1.75; CI = 1.01-3.32; p = 0.046; Figure 2). No association was seen between the 6-12 or 0-12 month trends in any other subset and molecular recurrence.

 In the large EUROSKI study of TKI withdrawal (Saussele et al 2018), musculoskeletal symptoms mimicking rheumatoid arthritis can arise in about a third of patients (Richter et al 2014). The mechanism of this TKI withdrawal syndrome is unknown. Of 34 assessable patients, 13 (38%) developed musculoskeletal symptoms of Common Terminology Criteria for Adverse Events grade 1 or 2 (no grade 3 or 4 symptoms were reported). The trends in CD4+ regulatory T cells and CD56dim and bright NK cells of Figure 1 showed no correlation with the presence of TKI withdrawal symptoms.

 In the present DESTINY study, we have recently shown that 72% of patients in stable MR4 before trial entry can complete their 36 month period of gradual TKI withdrawal without loss of MMR (Clark et al 2018). This result is superior to studies of outright TKI cessation, and suggests that this gradual withdrawal strategy may be responsible. The present study of lymphocyte subsets may provide some insight into the mechanism for this. Firstly, changes in CD4+ regulatory T cells, CD56dim and CD56bright NK cell subsets occur during TKI de-escalation; indeed no further changes are seen on stopping for any subset. Patients with higher levels of NK cells at treatment cessation have a higher probability of successful treatment discontinuation (Ilander et al 2017, Réa et al 2017); furthermore NK cell numbers increased further after treatment cessation in non-relapsing patients (Réa et al 2017). Here we extend these findings by showing that NK numbers may alter during treatment de-escalation, implying that the dose as well as the presence of TKI may be critical for determining TKI effect on the immune system.

 Secondly, a rise in the effector memory CD8+ subset predicts molecular recurrence. Higher CD86+ plasmacytoid dendritic cell counts correlate with a lower probability of successful treatment cessation and an exhaustion T cell phenotype (Schütz et al 2017). The net effect of TKIs on the immune system is clearly complex, but taken together the available and present data support the view that the immunological effect of TKIs may be more marked in patients who will relapse after treatment discontinuation. This clearly requires further study.

 Finally, we show no association between subset trends and musculoskeletal events on TKI withdrawal. To be clinically useful, a subset change predictive of relapse or troublesome withdrawal side effects would need to be apparent in a study of the present size, though further study in a larger population, together with functional studies, would be of interest.

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**SUPPLEMENTARY DATA**
 The following antibodies were used, all from BD Biosciences, Oxford, UK:

 BD Horizon V500 mouse Anti-Human CD45RA, clone 100, (#561640) BD Pharmingen, FITC Rat Anti-Human CCR7 (CD197), clone 3D12 (#560548)

 BD Pharmingen, PerCP-Cy 5.5 mouse Anti-Human CD25, clone M-A251 (#560503) BD Pharmingen, PE-Cy7 mouse Anti-Human CD127, clone HIL-7R-M21 (#560822)

 CD56 PE NCAM 16.2 (#345812)

 BD Horizon V450 mouse Anti-human CD4, clone RPA-T4 (#560345)

 BD Pharmingen, APC-H7 mouse Anti-Human CD27, clone M-T271 (#560222)

 BD Pharmingen, APC mouse Anti-human CD8, clone RPA-T8 (#555369)

 At room temperature, 100 µl of fresh ethylenediaminetetraacetic acid (EDTA) anticoagulated whole blood was added to each undiluted antibody (10µl of anti-CCR7 and CD56; 5µl of all others). After erythrocyte lysis using BD Lyse-Wash-Assistant and FACSLyse solution at 1:10 working concentration (BD Biosciences) and washing with phosphate buffered saline (PBS, Source BioScience, Rochdale, Greater Manchester, UK), ~1ml of 1% foetal bovine serum (Gibco by Life Technologies, Paisley, Renfrewshire, UK) and PBS was added to the resultant cell suspension.

 Samples were analysed by flow cytometry, using a FACSCanto II machine (BD Bioscience), and the resultant data were analysed by FACS Diva software, version 8.0.1.

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**LEGENDS to FIGURES and TABLES**

*Figure 1.*

Significant changes in lymphocyte subsets with time. Results are expressed as mean +/- standard error. Panel A = regulatory CD4+ T cells; B = CD56dim NK cells; C = CD56bright NK cells. Significance levels are compared with baseline using the Mann-Whitney test in each case.

*Figure 2.*

Time trends in effector memory CD8+ cells, stratified by molecular relapse. Values are means +/- standard error. No 18 month data are available, as all patients who relapsed had done so and therefore resumed full dose TKI before reaching this time point. The association between central memory subsets (whether CD4+ or CD8+) and molecular recurrence could not be assessed as these were undetectable in several patients at several time points. Details of the odds ratio, confidence intervals and significance levels are given in the text.

*Supplementary Table 1.*

Demographic, treatment and outcome details of the study population. UPN = unique patient number. ‘Completed’ = reached the end of the study at month 36 without molecular relapse. Patients whose outcome is asterisked had an atypical outcome, described in the text.

Supplementary Table 1. Demographic, treatment and outcome details of the study population.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **UPN** | **Sex/Age** | **Transcript Type** | **HLA Class I** | **Entry TKI** | **Outcome** |
| 01 | F65 | e14a2 | A2, A3; B7, B44 | Nilotinib | Completed |
| 02 | M62 | e13a2 | A2, - ; B13, B51 | Nilotinib | Completed |
| 03 | M57 | e14a2 | A3, A68; B35, B41 | Imatinib | Relapse month 14 |
| 04 | F71 | e13a2 + e14a2 | A1, A2; B37, B44 | Nilotinib | Stopped month 5\* |
| 05 | F69 | e13a2 | A2, A24; B7, B44 | Nilotinib | Relapse month 18 |
| 06 | M64 | e14a2 | A1, A2; B37, B44 | Imatinib | Completed |
| 07 | F53 | e14a2  | A2, A24; B7, B44 | Imatinib | Completed |
| 08 | M52 | e14a2 | A3, A32; B27, - | Imatinib | Relapse month 8 |
| 09 | M60 | e14a2 | A2, A26; B7, B51 | Imatinib | Relapse month 14 |
| 10 | F52 | e13a2 | A2, A25; B18, B40 | Nilotinib | Completed |
| 11 | M56 | e13a2 + e14a2 | A2, - ; B15, B41 | Imatinib | Completed |
| 12 | M56 | e14a2 | A2, A3; B7, B18 | Imatinib | Completed |
| 13 | M46 | e14a2 | A1, A24; B44, - | Imatinib | Relapse month 14 |
| 14 | F65 | e14a2 | A1, A23; B8, B49 | Nilotinib | Completed |
| 15 | F70 | e14a2 | A1, A23; B15, B44 | Imatinib | Relapse month 14 |
| 16 | F75 | e13a2 | A26, A30; B44, B57 | Imatinib | Relapse month 14 |
| 20 | F60 | e14a2 | A2, A24; B7, B44 | Imatinib | Completed |
| 21 | M56 | e14a2 | A24, A31; B8, B51 | Imatinib | Completed |
| 23 | F64 | e13a2 | A1, A2; B8, B15 | Imatinib | Relapse month 7 |
| 26 | F68 | e14a2 | A1, A3; B8, B15 | Imatinib | Relapse month 14 |
| 28 | M66 | e14a2 | A2, - ; B7, B62 | Imatinib | Relapse month 8 |
| 29 | M69 | e13a2 | A2, A3; B7, B62 | Imatinib | Completed |
| 35 | F64 | e14a2 | A1, - ; B8, - | Nilotinib | Completed |
| 37 | F78 | e14a2 | A2, A23; B56, B65 | Imatinib | Completed |
| 38 | M35 | e14a2 | A26, A30; B18, B27 | Imatinib | Relapse month 13 |
| 46 | M70 | e13a2 | A68, - ; B41, B65 | Dasatinib | Relapse month 13 |
| 54 | M49 | e14a2 | A2, A3; B44, B47 | Imatinib | Completed |
| 55 | M57 | e13a2 | A2, A29; B44, -  | Imatinib | Relapse month 35 |
| 61 | M57 | e14a2 | A1, A2; B8, B27 | Dasatinib | Relapse month 15 |
| 69 | M69 | e13a2 | A3, A30; B7, B13 | Imatinib | Completed |
| 80 | F55 | e14a2 | A2, - ; B7, B44 | Dasatinib | Relapse month 14 |
| 86 | F31 | e13a2 | A2, A24; B49, B62 | Dasatinib | Stopped month 7\* |
| 101 | F70 | e13a2 | A3, - ; B44, B65 | Nilotinib | Resumed month 16\* |
| 104 | M53 | unknown | A1, A29; B44, B57 | Imatinib | Relapsed month 15  |
| 108 | M68 | e19a2 | A2, A24; B60, B62 | Imatinib | Completed |
| 110 | F61 | e14a2 | A3, A68; B64, -  | Nilotinib | Completed |
| 143 | F73 | e14a2 | A2, A3; B8, B65 | Imatinib | Relapse month 14 |
| 144 | F62 | e14a2 | A1, A3; B7, B44 | Imatinib | Relapse month 14 |
| 156 | M57 | unknown | A1, A11; B8, B51 | Imatinib | Completed |

Figure 1.

Figure 2.