Patterns and kinetics of T-cell chimerism after allo transplant with alemtuzumab-based conditioning: mixed chimerism protects from GVHD, but does not portend disease recurrence

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Abstract
We analyzed the kinetics of CD3 chimerism in 120 consecutive allogeneic hematopoietic cell transplantation (HCT) recipients receiving alemtuzumab-based conditioning. Fifty-two received fludarabine/melphalan, 44 received fludarabine/busulfan, and 24 received clofarabine/melphalan in addition to alemtuzumab. Post-transplant GVHD prophylaxis consisted of tacrolimus. No prophylactic donor lymphocyte infusion or other interventions were used for mixed donor chimerism (MDC). Bone marrow (BM) and/or peripheral blood (PB) samples were obtained at 30 days, 100 days, 180 days, and 1 year following HCT. On Day 30, 15% of assessable patients had MDC in the CD3 compartment. This had increased to 50% by Day 100, and to 63% by Day 180. MDC predicted for a lower risk of acute ($p = 0.08$) and particularly of chronic GVHD ($p = 0.01$). MDC was not associated with subsequent relapse or TRM ($p = 0.67$ and 0.72, respectively). A decline of more than 15% in CD3 chimerism between Day 30 and Day 180 predicted for a 40% risk of subsequent disease recurrence. The observation of MDC after alemtuzumab conditioning does not by itself constitute a risk factor for relapse and should not be used to guide therapeutic intervention. By contrast, declining donor chimerism between Day 30 and Day 180 is associated with a somewhat increased risk of disease recurrence. The high incidence of MDC after alemtuzumab containing conditioning contributes to the low risk of acute and chronic GVHD.

Keywords: Allogeneic transplant, chimerism, GVHD, alemtuzumab

Introduction
Donor chimerism following allogeneic hematopoietic stem cell transplantation (HCT) is useful to determine engraftment and has been increasingly used to predict transplant outcomes [1]. Very low levels of donor chimerism, particularly of T- and NK-cells are associated with increased risk of graft rejection and poor outcomes [1–4]. Complete donor T-cell chimerism or rapid achievement of complete donor T-cell chimerism has been associated with an increased risk of GVHD [1,5–8]. Persistent or protracted mixed T-cell chimerism, on the other hand, is associated with a reduced, but not absent risk for GVHD in animal models and in human transplant, as is delayed attainment of full T-cell chimerism [9–15]. Several groups have also found an increased risk of disease recurrence with persistent mixed chimerism of T-cells [5,16] and studies have been conducted or are underway investigating the use of prophylactic donor lymphocyte infusion (DLI) for persistent or declining mixed T-cell chimerism [15,17,18]. But others have reported excellent disease responses in patients with mixed chimerism [16,19]; and in some studies protracted mixed chimerism was associated with improved outcome.
although the interpretation of results tended to be complicated by the use of prophylactic DLI [10,15]. We have studied conditioning regimens incorporating alemtuzumab in combination with a variety of chemotherapy regimens. We previously reported our data on chimerism analyzed in unfractionated cells, consisting mainly of myeloid progenitors. We found a very high percentage of donor chimerism (median 93% on Day 30 and 88% on Day 100) that was relatively stable over time [20]. We also found a strong correlation between bone marrow (BM) and peripheral blood (PB) chimerism. PB chimerism on Day 30 or Day 100 did not predict for transplant outcomes such as GVHD or relapse [20]. Lim et al. [21] suggested that lineage specific analysis of chimerism might be more informative, though their own analysis was confounded by the use of prophylactic DLI for patients with mixed donor chimerism (MDC) in the lymphoid lineage. Here we report our analysis of the relation between CD3-selected cell chimerism and transplant outcomes in a subsequent cohort of patients. In contrast to others, we chose not to intervene for mixed chimerism. This allowed us to study the relation between chimerism and HCT outcomes after alemtuzumab-prepared transplants without any confounders.

Patients and methods

Patients and treatment

Lineage-specific T-cell (CD3) chimerism was assayed on 120 consecutive patients with hematologic malignancies receiving alemtuzumab-based conditioning who survived to at least Day 25 after transplant. Baseline characteristics are listed in Table I. All patients signed written informed consent for institutional review board approved prospective studies. The median age of this group was 56 years (range, 18–71).

Conditioning regimens included fludarabine, melphalan, alemtuzumab (n = 52) [22] fludarabine, busulfan, alemtuzumab (n = 44) [23]; and clofarabine, melphalan, alemtuzumab (n = 24) [24]. These regimens have all been previously reported and included identical doses of alemtuzumab. Patients participating in an earlier Phase I dose escalation study of clofarabine-melphalan were not included in this analysis, because the low doses of clofarabine and melphalan in the initial patients might have interfered with engraftment [24]. Filgrastim mobilized PB stem cells were utilized for all related donor transplants and were routinely requested for unrelated donor transplants. Post-transplant immunosuppression consisted of tacrolimus administered IV beginning Day-2 and then orally from engraftment until at least Day 100. In recipients of an human leukocyte antigen (HLA)-identical sibling, tacrolimus was tapered over a period of 2–3 weeks, starting on Day 100, unless there was evidence of acute or chronic GVHD. In recipients of unrelated or mismatched transplant, tacrolimus taper was started on Day 180. Immunosuppression was withdrawn early if overt disease relapse occurred.

Patients were transplanted over a 50 month period between December 2004 and February 1, 2009. Data were analyzed with follow-up through March 15, 2009. The median duration of follow-up for surviving patients was 16 months (range, 1–51 months).

Definitions

HLA matching was based on high-resolution at eight loci (HLA-A, B, C, and DRB-1). Disease risk followed the American Society for Blood and Marrow Transplantation criteria [25]. Acute and chronic GVHD were diagnosed and scored according to standard methods, although the consensus definition of chronic GVHD was not yet utilized in this analysis [26,27]. Suspected GVHD was confirmed by biopsy whenever possible. Relapse was defined as overt relapse with clinical, pathological, or radiological signs or symptoms. Progression free survival time was defined as the time from transplant until relapse or death, whichever happened earlier. Transplant related mortality was any death not caused or preceded by relapse.
**Chimerism analysis**

BM and/or PB samples were obtained at ~30 days, 100 days, 180 days, and 1 year following HCT. Samples were then collected yearly thereafter, and at the time of disease recurrence.

CD3-positive cells were purified by a manual procedure using biotinylated antibody and avidin-labeled magnetic particles (EasySep CD3 cell separation kit, Stem Cell Technologies). A purity of ≥95% CD3 positive cells was routinely achieved using this procedure. DNA was extracted from both unfractionated samples and CD3(+) cells using QiaAmp™ kits (Qiagen). DNA concentration and purity were measured using a Nanodrop™ spectrophotometer.

Chimerism was analyzed using short tandem repeat (STR) analysis using the Profiler Plus Kit™ (ABI), a multiplex PCR reaction which simultaneously amplifies 10 STR loci. The protocol was modified to analyze more DNA (20 ng per reaction) than in the intended forensic application. Reaction products were measured on an ABI3100 capillary electrophoresis analyzer. This assay was shown to be linear for mixtures in the range of 5–95%. Sensitivity was reliable down to 5%, occasionally to 2–3%. Hence, a result of ≥95% was considered full donor chimerism (FDC). Detectable donor CD3 chimerism less than 95% was considered MDC.

**Chimerism kinetics.** Chimerism change was assessed at Day 30, Day 100, Day 180, and Day 360. A threshold of 15% was chosen to represent a change in chimerism because it represented three intervals from the limit for sensitivity of the assay (i.e., 5%). As such, chimerism was considered stable, if from one time point to the next one there was less than 15% absolute change. It was considered declining if there was ≥15% decline between time points.

**Statistical analysis**

Chimerism. We evaluated the agreement between CD3-selected cells and unfractionated chimerism using two way scatter plots and correlation analysis. We then analyzed PB and BM chimerism using the same methodology and confirmed a close correlation between PB and BM CD3+ chimerism with correlation coefficients ranging from 0.67 to 0.94 at the various time points.

We did not analyze unfractionated chimerism further because our previous analysis had already shown a lack of predictive power. PB chimerism was used for the vast majority of datapoints for the CD3 chimerism analysis. Out of 315 data points, 287 were based on measured PB CD3 data. For 23 data points we used a corresponding BM measure. For the remaining five data points, neither PB nor BM chimerism was available at a particular time point, but chimerism was available for preceding and subsequent time points, was stable during the interval and, therefore, was imputed from preceding and subsequent values.

Time to event models used the day of transplant as Day 0. We used Cox Proportional Hazards analysis (results not shown) to analyze the effect of chimerism after adjusting for the effects of covariates. To ensure chimerism results predicted future events, we excluded patients who had an event 10 days or less after the chimerism timepoint was measured in time to event models.

Analyses for time to acute GVHD, chronic GVHD, relapse, or death are subject to competing risks and were analyzed using Gray’s test [28,29]. We also performed an alternative analysis using fixed time periods to confirm the above analysis and analyze subsets. In this method, we define in advance a time period after transplant during which the event of interest will be monitored. Patients who experience the event during this period are considered a “success” and patients who are under study for the entire period but do not experience the event are a “failure”. Patients who do not experience the event and are not followed for the entire time period are not evaluable. In addition, patients who experience a competing risk event prior to the event under study are not evaluable. These data can be summarized as a 2 by 2 table (e.g., Predictor:FDC/MDC vs. event: relapse/not relapse) and the effect of predictor on outcome event tested by the $\chi^2$ test. The results obtained with the fixed time period method were consistent with those obtained by Gray method. The fixed time period method was also used for an analysis of the impact of acute and chronic GVHD on relapse.

**Results**

**Peripheral blood CD3 chimerism, relation of peripheral blood with bone marrow chimerism, and relation between CD3 chimerism and unfractionated chimerism (Table II)**

Median donor CD3 chimerism was 95% by Day 30 and only 15% of recipients had MDC. The median donor CD3 chimerism declined over time leading to a higher proportion of MDC.

There were no differences in patterns of chimerism after fludarabine busulfan or fludarabine melphalan (Table III). Clofarabine melphalan had a significantly lower percentage of MDC on Day 100 and Day 180 compared fludarabine melphalan. There was also a significantly lower percentage of MDC in recipients of unrelated/mismatched transplant. Underlying
diagnosis (AML/MDS vs. NHL/HD) was not associated with differences in CD3 chimerism.

Figure 1 (a) depicts the evolution of PB CD3+ donor chimerism in all patients. Figure 1 (b) shows the evolution of unfractionated (i.e., myeloid) chimerism. CD3 chimerism was almost always lower than unfractionated chimerism and tended to decline more frequently. The relationship between CD3 chimerism and myeloid chimerism is further illustrated in Figures 2 (a) and 2(b).

CD3 chimerism and transplant outcome

Chimerism and cumulative incidence of GVHD and relapse. Figure 2 depicts the impact of Day 30 MDC on the cumulative incidence of a GVHD, relapse, and TRM as well as the influence of Day 100 MDC on cGVHD. MDC on Day 30 was associated with a reduced risk for acute GVHD ($p = 0.08$). MDC on Day 100 was associated with a significantly reduced risk of chronic GVHD ($p = 0.01$). No significant difference in risk for relapse or TRM was observed between those with MDC or FDC on Day 30. Similarly, Day 100 chimerism did not impact relapse rates or TRM (data not shown).

Fixed time period analysis and GVHD (Table IV). Day 30 chimerism was analyzed in 83 patients who survived without relapse until Day 100. None of 13 (0%) with MDC on Day 30 developed acute GVHD by Day 100 compared with 17 of 70 (24%) with FDC ($p = 0.046$). Among the 59 evaluable patients who survived without relapse until Day 365, 3 of 32 (9%) with MDC at Day 100 developed chronic GVHD compared with 14 of 27 (52%) with FDC ($p < 0.001$).

Fixed time period analysis and relapse (Table IV). The effect of Day 30 chimerism on Day 100 relapse was

Table II. Peripheral blood chimerism and evolution of peripheral blood chimerism over time.

<table>
<thead>
<tr>
<th>N evaluable</th>
<th>Median percentage donor chimerism CD3 (range)</th>
<th>N with mixed donor chimerism (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 30</td>
<td>120</td>
<td>95 (16–96)</td>
</tr>
<tr>
<td>Day 100</td>
<td>98</td>
<td>94.5 (0–96)</td>
</tr>
<tr>
<td>Day 180</td>
<td>66</td>
<td>88 (9–96)</td>
</tr>
<tr>
<td>Day 365</td>
<td>31</td>
<td>61 (7–96)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Median % decline in CD3 chimerism from Day 30 (range)</th>
<th>N with declining donor chimerism (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 100</td>
<td>98</td>
</tr>
<tr>
<td>Day 180</td>
<td>66</td>
</tr>
<tr>
<td>Day 365</td>
<td>31</td>
</tr>
</tbody>
</table>

*Full donor chimerism: ≥95% donor chimerism in CD3 lineage.
†Declining chimerism: ≥15% absolute decline between Day 30 and day of assessment.

<table>
<thead>
<tr>
<th>Conditioning regimen</th>
<th>% MDC d30</th>
<th>%MDC d100</th>
<th>%MDC d180</th>
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<tr>
<td>Fludarabine melphalan</td>
<td>52</td>
<td>19</td>
<td>60</td>
</tr>
<tr>
<td>Fludarabine busulfan</td>
<td>44</td>
<td>11</td>
<td>47</td>
</tr>
<tr>
<td>Clofarabine melphalan</td>
<td>24</td>
<td>8</td>
<td>31**</td>
</tr>
</tbody>
</table>

**p = 0.026 compared with fludarabine melphalan conditioning.
***p = 0.040 compared with fludarabine melphalan conditioning.
††p = 0.015 compared with matched related.
†††p = 0.026 compared with matched related.
††††p = 0.028 compared with matched related.

<table>
<thead>
<tr>
<th>Donor source</th>
<th>N*</th>
<th>% MDC d30</th>
<th>%MDC d100</th>
<th>%MDC d180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matched related</td>
<td>59</td>
<td>22</td>
<td>61</td>
<td>74</td>
</tr>
<tr>
<td>Mismatched or unrelated</td>
<td>61</td>
<td>7†</td>
<td>38††***</td>
<td>51††***</td>
</tr>
</tbody>
</table>

Diagnosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>% MDC d30</th>
<th>%MDC d100</th>
<th>%MDC d180</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML/MDS</td>
<td>74</td>
<td>15</td>
<td>54</td>
</tr>
<tr>
<td>NHL/HL</td>
<td>28</td>
<td>11</td>
<td>41</td>
</tr>
<tr>
<td>Others</td>
<td>18</td>
<td>17</td>
<td>47</td>
</tr>
</tbody>
</table>

MDC, mixed donor chimerism.
*N = number evaluable on Day 30. For later time points, fewer patients are evaluable.
**p = 0.026 compared with fludarabine melphalan conditioning.
***p = 0.040 compared with fludarabine melphalan conditioning.
†p = 0.015 compared with matched related.
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tested among 109 evaluable subjects. Two of 17 (12%) with MDC relapsed compared with 9 of 92 (10%) with FDC ($p = 0.80$). Among the 49% with high disease risk, relapse occurred in 1 of 10 (10%) with Day 30 MDC compared with 6 of 44 (14%) with FDC ($p = 0.76$). Among the 50% with low/intermediate disease risk patients, one of seven with MDC relapsed compared with 3 of 48 with FDC ($p = 0.44$).

The impact on relapse at Day 365 for Day 100 chimerism was assessed in 63 patients. Relapse occurred in 10 of 33 with MDC compared with 12 of 30 (40%) with FDC ($p = 0.42$). For high disease risk patients, relapse occurred in 7 of 15 (47%) with MDC compared with 9 of 15 (60%) with FDC ($p = 0.46$). Among low/intermediate risk patients, 3 of 18 (17%) with Day 100 MDC relapsed by Day 365 versus 3 of 15 (20%) of those with FDC ($p = 0.80$).

### Declining chimerism and relation to outcome

We dichotomized CD3 change into stable and declining using a threshold of 15% change in CD3 chimerism from Day 30 compared with subsequent timepoints as shown in Table II. Declining CD3...
chimerism from Day 30 was found in 26 of 98 (26%) by Day 100, 27 of 66 (41%) by Day 180, and 19 of 31 (61%) by Day 365.

Declining chimerism by Day 100 was not associated with an increased risk for relapse ($p = 0.65$). By contrast, declining chimerism from Day 30 to Day 180 showed a significantly increased risk of relapse ($p = 0.05$) (Figure 3). Specifically, around 40% of those with declining chimerism by Day 180 relapsed compared with 10% of those with stable chimerism.

**Acute and chronic GVHD and disease recurrence**

The impact on relapse by Day 365 for those with acute GVHD by Day 100 was assessed in 70 patients. Relapse occurred in 2 of 12 with acute GVHD and in 22 of 58 without acute GVHD ($p = 0.15$). The impact on relapse for those with chronic GVHD by Day 180 was assessed in 59 patients. Relapse occurred in 1 of 11 with chronic GVHD and in 15 of 33 without chronic GVHD ($p = 0.14$).

**Discussion**

In animal models, permanent MDC is associated with reduction in GVHD because of induction of a state of donor/recipient tolerance [12]. In humans, the degree of chimerism after transplant depends on factors, such as the amount of prior chemotherapy, prior transplant, donor source, graft composition, conditioning regimen, and method of GVHD prophylaxis [1]. MDC is often observed, but has been difficult to reliably induce. Herein, we report the natural history and impact on post-transplant outcomes of CD3 chimerism kinetics after alemtuzumab-based conditioning.

The large majority of our patients initially achieved complete or nearly complete chimerism in the CD3 and in the unfractionated compartment. Unfractionated and, particularly, CD3 chimerism declined over time and many patients have developed a state of stable CD3 MDC. This pattern of CD3 MDC is radically different from that observed after reduced intensity conditioning without alemtuzumab [1–4]. For example, the Seattle group reported after nonmyeloablative conditioning an early high rate of mixed chimerism [16]. Over time, these grafts were either rejected or chimerism increased. Stable mixed chimerism is rare.

We used an intensive conditioning regimen consisting of a high dose alkylating agent (either melphalan or busulfan) combined with a nucleoside analog (fludarabine or clofarabine). These combinations are extremely myelosuppressive and immunosuppressive probably because of synergy between the two classes of agents [30], and explain the high early rate of full chimerism and the absence of graft rejection. Valcarcel et al. [31], reported that busulfan in particular was less immunosuppressive than melphalan. In our analysis the kinetics and pattern of chimerism did not depend on the use of melphalan or busulfan and, therefore, results should be mainly attributed to an alemtuzumab effect. Of interest, those who received conditioning including clofarabine had somewhat lower rates of MDC, confirming the potent immunosuppressive effect of clofarabine [24].

The patterns of chimerism reported by Lim et al. [15] who used fludarabine, reduced dose busulfan, and alemtuzumab are informative. They too observed a high incidence of mixed chimerism in the T-cell lineage. Lim et al. also observed that in those with MDC, chimerism often declined by Day 100, and in those cases they routinely intervened with prophylactic DLI, obscuring conclusions about the influence of MDC in their patients. They, like us, observed a significantly higher percentage of FDC in the group of unrelated or mismatched transplants, an observation that is difficult to explain.

![Figure 3](https://www.leuklymph.org/article/image/leuklymph-10-07-11-figure3.png)

Figure 3. Time to relapse based on chimerism kinetics (a) Chimerism between Day 30 and Day 100: solid line stable; dashed line declining. (b) Chimerism between Day 30 and Day 180 solid line: stable chimerism. dashed line: declining chimerism.
Interestingly, all cases of acute GVHD occurred in those with FDC by Day 30 and nearly all cases of chronic GVHD occurred in those with FDC at Day 100. In other words, FDC was, as in other studies, a risk factor for both acute and chronic GVHD [1,5–8]. But the cumulative risk of acute and chronic GVHD in those with MDC was extremely low. This is again consistent with the analysis by Lim et al. Mixed chimerism after alemtuzumab conditioning is more protective of GVHD, than after other conditioning regimens [19,32], perhaps because it tends to be stable or declining over time, and rarely increasing.

The most controversial implication of mixed chimerism is its possible association with disease recurrence. Some groups have shown an increased relapse rate with MDC [5,16]. Childs et al. [5], studied 15 patients and found that increasing chimerism preceded responses in many of them. Importantly though, most of the patients in this small study had melanoma or renal cell cancer, diseases that are notoriously refractory to chemotherapy. Mohty et al. [33], analyzed the kinetics of CD3\(^+\)-T-cell chimerism in 41 patients with myeloid malignancies. The delayed establishment of FDC in 15 patients was associated with a higher incidence of relapse (40% vs. 0; \(p = 0.002\)), but this analysis was not adjusted for TRM, an important competing risk for disease recurrence and commonly increased among those with GVHD. An interesting study from Keil et al. [34] found a very high rate of relapse in patients who, on Day 28 after reduced intensity conditioning, had less than 90% T-cell chimerism. However, many of the relapses occurred within 3 months after transplant and it is likely that, in this case, MDC was more indicative of relapse than predictive. Baron et al. [1], found that FDC after nonmyeloablative transplant, was associated with decreased risk for relapse. The impact on progression free survival was less clear. The retrospective nature of these studies, the limited patient numbers in some of them and the methodological problems for others, leave considerable doubt over the conclusions, particularly because other studies have shown an improved long-term outcome with MDC [10,15]. To avoid toxicity [15,35,36], we did not use prophylactic DLI. We previously reported that we did not find evidence that MDC in unfractionated cells predicted for relapse and here we failed to find a relationship between CD3 chimerism and risk of disease recurrence, even after adjusting for other risk factors for relapse. Twenty patients in this series, remain in remission for more than 2 years after transplant, ranging from 24 to 51 months. For this group of patients, the median donor PB CD3 chimerism at 1 year after transplant is 73% (19–95%) and only three patients have FDC. Our analysis thus indicates that, at least after an alemtuzumab containing regimen, MDC in CD3 cells does not predict for increased risk of disease recurrence. Many patients with stable MDC do very well for prolonged periods of time and should not be exposed to the risks of further therapy with DLI or other interventions. The latter conclusion pertains particularly to patients with myeloid malignancies who constituted the majority of patients in this series. It remains possible that the effects of mixed chimerism are different in other malignancies, particularly some subtypes of lymphoma. Overt GVHD by contrast was associated with a somewhat decreased risk of disease recurrence. That reduction was not statistically significant and was offset by an increased risk for treatment related mortality.

Although stable mixed T-cell chimerism did not predict for relapse, declining CD3 chimerism did. The practical value of the association between declining CD3 chimerism on Day 180 and subsequent recurrence is unfortunately limited because many relapses have already occurred by Day 180. Still, further studies could be designed that use declining chimerism as a trigger for intervention to prevent clinical recurrence. One could for example envisage withdrawal of immunosuppression and/or DLI for patients with declining donor chimerism [37]. In contrast, those with FDC may benefit from prolonged immunosuppression to abrogate the risk of GVHD and potentially induce MDC.

In summary, this series confirms that alemtuzumab, combined with a nucleoside analog and an alkylating agent, results in a very high incidence of Day 30 full donor CD3 chimerism. Over time, the percentage of patients with MDC increases. After alemtuzumab-based conditioning, CD3 MDC on Day 30 and Day 100 is a powerful predictor of near absence of risk for GVHD. Stable CD3 MDC was not associated with an increased risk of disease recurrence. Prophylactic DLI for mixed chimerism would likely have caused an increased incidence of GVHD and should not be recommended in this setting. Declining mixed chimerism on the other hand suggests a somewhat increased risk for relapse and may better guide therapy. We believe the ideal situation for our patients would be the achievement of a state of stable CD3 MDC which is protective of both GVHD and disease recurrence. Further studies are ongoing to identify predictors of this outcome.

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Koen van Besien: designed research, performed research, analyzed data, wrote manuscript. Alexander Dew: analyzed data, wrote manuscript. Shang Lin: analyzed data, wrote manuscript. Loren Joseph: performed engraftment analysis, reviewed


