Supplementary

Materials

- Culture medium (RPMI-1640, Gibco, with 2% FBS and 1% of L-glutamine, 1% penicillin and streptomycin)
- Wash medium (RPMI-1640 with 2% FBS and 1% of L-glutamine, 1% penicillin and streptomycin, Gibco)
- Freezing medium (RPMI-1640, Gibco, with 10% FBS and 1% of L-glutamine, 1% penicillin, streptomycin and 10% DMSO)
- Round-bottom 96-wells plates (sterile, non-treated, Nunc, Thermo Fisher Scientific)
- GolgiStop (BD Biosciences,)
- PBS (Gibco)
- FACS buffer (PBS, 0,1% NaN3, 2% FBS)
- Mouse and Rat serum
- Live/Dead cell viability dye (efluor506)
- Brilliant Stain buffer (Invitrogen Thermo Fisher Scientific)
- Rainbow calibration particles (Brand)
- For all staining antibodies used see in supplementary table X
- Fixation and Permeabilization kit (Fix/Perm, Perm buffer, Invitrogen Thermo Fisher Scientific)
    - Fixation and Permeabilization reagent (Fixation/Permeabilization concentrate and diluent in ratio 1:3)
    - Perm buffer (Permeabilization buffer and demi water in ratio 1:10)

Supplementary data

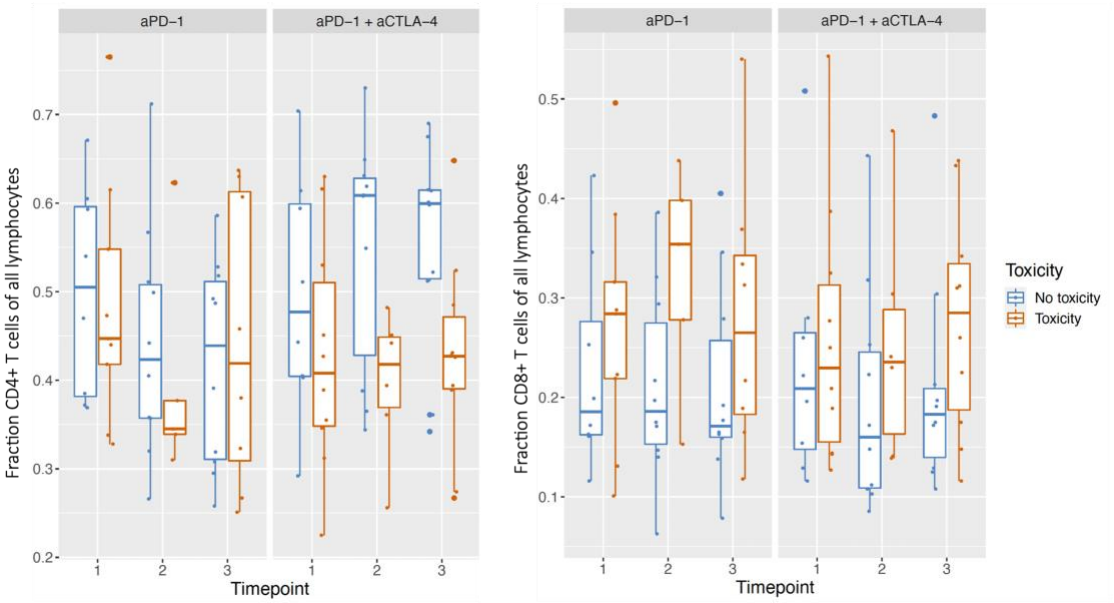

Figure 1. In all boxplots, the medians with interquartile range is shown. CD4+ and CD8+ T cells as a part of all lymphocytes (live CD3+) were measured before treatment (baseline)(Timepoint = 1), after the first (Timepoint = 2) and second cycle of treatment (Timepoint = 3) or when toxicity occurred (Timepoint = 3). Also stratified per treatment (anti-PD1 or anti-PD1 + anti-CTLA-4 treatment)

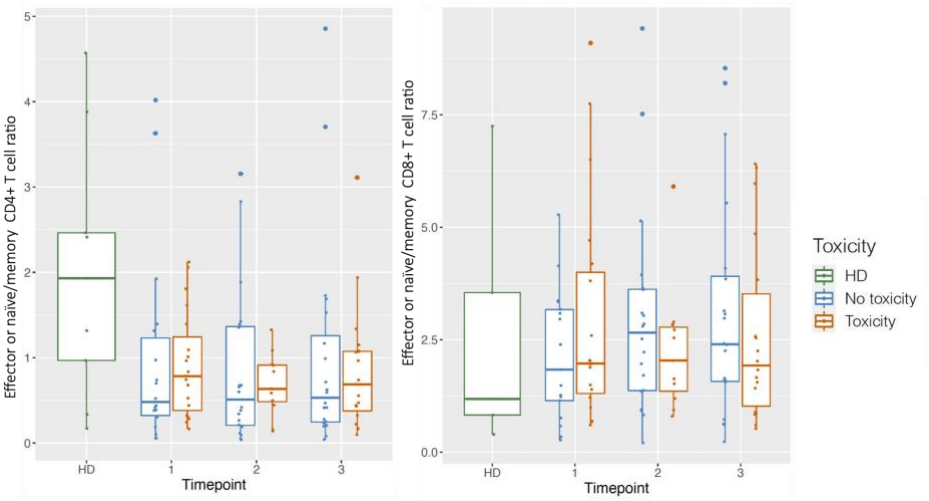

Figure 2. Effector or naïve or naïve / memory ratio (CD45RO- /CD45RO+) of CD4 and CD8+ T cells.

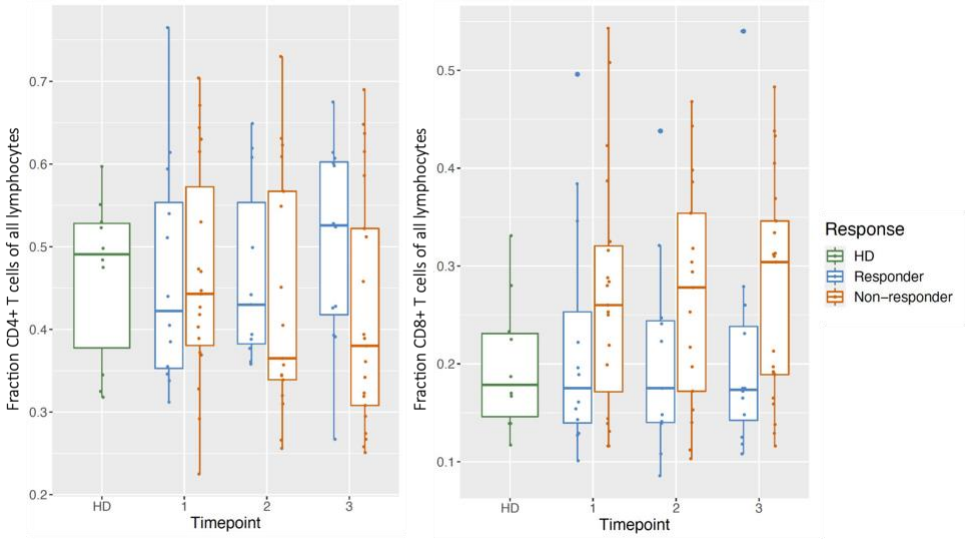

Figure 3. B. CD4+ and CD8+ T cells as a part of all lymphocytes (live CD3+) for responders and non-responders.

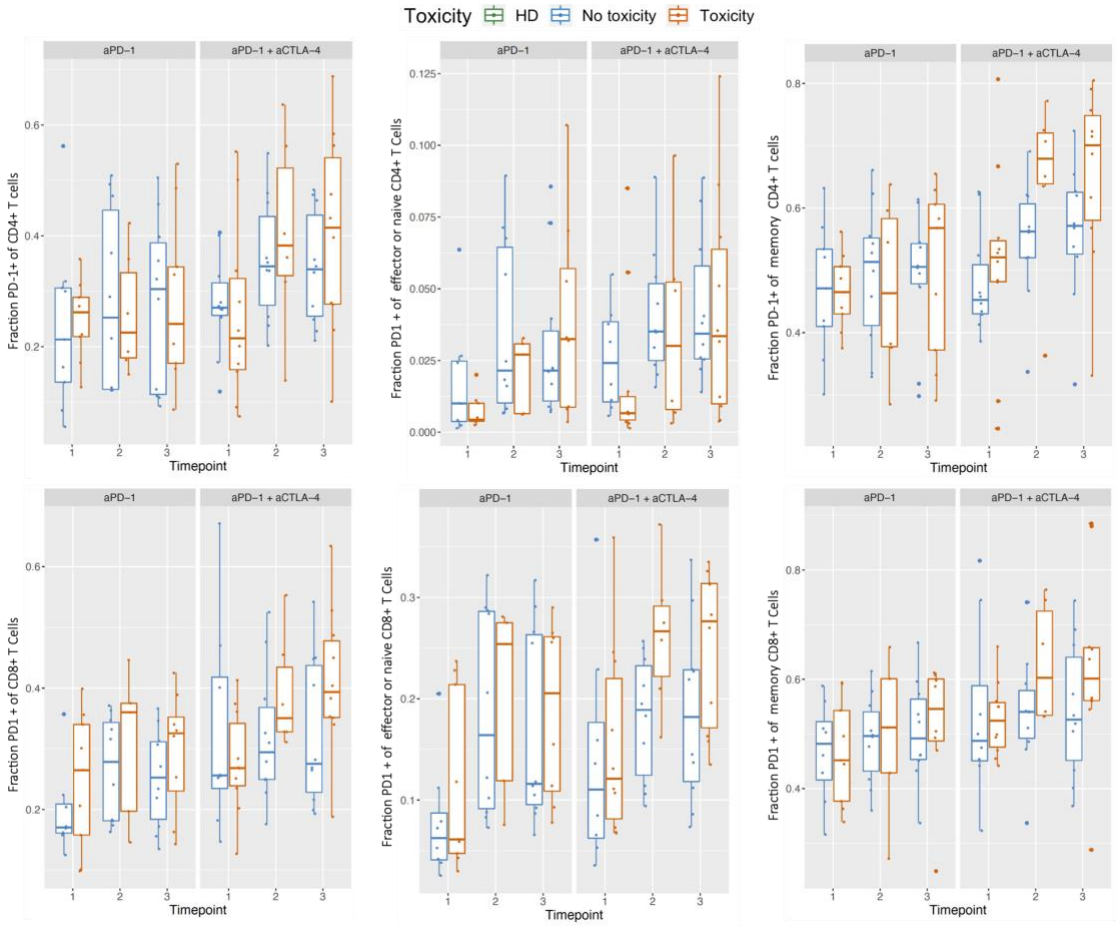

Figure 4. Fractions PD-1+ of CD4+ and CD8+ T cells, also within effector or naïve (CD45RO-) and memory (CD45RO+) subsets for patients with and without toxicity measured at all time points, stratified per treatment type.

Statistics

Examples in R studio

Mixed ANOVA, Repeated measures ANOVA, Wilcoxon-rank test

Panel 2 Toxicity VS no Toxicity on multiple timepoints

P_2_PD1pos_ of_CD4mem (PD1+ fraction of memory CD4+ T cells)

#Mixed ANOVA

```
> mixed_anova_P_2_PD1pos_of_CD4mem <- aov_ez(id = "P_UNI",
                    dv = "P_2_PD1pos_of_CD4mem",
                    data = subset(Panel2_possiblevals, c(P_tox != "HD" & P_time != 2)),
                    between= "P_tox",
                    within = "P_time",na.rm=TRUE)
```

Output

```
> mixed_anova_P_2_PD1pos_of_CD4mem
Anova Table (Type 3 tests)

Response: P_2_PD1pos_of_CD4mem
        Effect    df  MSE        F  ges p.value
1       P_tox 1, 36 0.02     0.98 .019    .329
2      P_time 1, 36 0.01 10.87 ** .080    .002
3 P_tox:P_time 1, 36 0.01     0.79 .006    .380
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘+’ 0.1 ‘ ’ 1
```

#Repeated-measures anova to check in which group the significant effect for time is

```
> repeated_measures_mixed_anova_P_2_PD1pos_of_CD4mem <-
  subset(Panel2_possiblevals, c(P_tox != "HD" & P_time != 2)) %>%
   group_by(P_tox) %>%
  anova_test(dv = P_2_PD1pos_of_CD4mem, wid = P_UNI, within = P_time) %>%
  get_anova_table() %>%
  adjust_pvalue(method = "bonferroni")
```

Output

```
> repeated_measures_mixed_anova_P_2_PD1pos_of_CD4mem
# A tibble: 2 x 9
  P_tox       Effect   DFn   DFd     F     p `p<.05`   ges p.adj
  <chr>       <chr>  <dbl> <dbl> <dbl> <dbl> <chr>   <dbl> <dbl>
1 No toxicity P_time     1    18  3.02 0.099 ""      0.062 0.198
2 Toxicity    P_time     1    18  8.43 0.009 "*"     0.098 0.018
```

#Effect in time is within the toxicity group. Wilcoxon-rank test was done to see where the difference is.

#Make a subset with wanted variables

```
> Susbset_P_2_PD1pos_of_CD4mem <- subset(Panel2_possiblevals,c(P_tox != "HD"))
  %>% select(P_2_PD1pos_of_CD4mem, P_tox, P_time, P_UNI)
```

Subset wide instead of long

```
> Susbset_P_2_PD1pos_of_CD4mem_wide <-Susbset_P_2_PD1pos_of_CD4mem %>%
  pivot_wider(names_from = "P_time", names_prefix= "PD1_CD4mem_",
                    values_from = P_2_PD1pos_of_CD4mem, values_fill = NA)
```

#Calculate change scores

```
> Susbset_P_2_PD1pos_of_CD4mem_wide$change12 <-
  Susbset_P_2_PD1pos_of_CD4mem_wide$PD1_CD4mem_2-
  Susbset_P_2_PD1pos_of_CD4mem_wide$PD1_CD4mem_1

> Susbset_P_2_PD1pos_of_CD4mem_wide$change13 <-
  Susbset_P_2_PD1pos_of_CD4mem_wide$PD1_CD4mem_3-
  Susbset_P_2_PD1pos_of_CD4mem_wide$PD1_CD4mem_1

> Susbset_P_2_PD1pos_of_CD4mem_wide$change23 <-
  Susbset_P_2_PD1pos_of_CD4mem_wide$PD1_CD4mem_3-
  Susbset_P_2_PD1pos_of_CD4mem_wide$PD1_CD4mem_2
```

```
# Perform Wilcoxon-rank tests
> wilcox.test(change12 ~ P_tox,
  data = Susbset_P_2_PD1pos_of_CD4mem_wide,
  paired = FALSE,
  alternative = "two.sided")

> wilcox.test(change13 ~ P_tox,
  data = Susbset_P_2_PD1pos_of_CD4mem_wide,
  paired = FALSE,
  alternative = "two.sided")

> wilcox.test(change23 ~ P_tox,
  data = Susbset_P_2_PD1pos_of_CD4mem_wide,
  paired = FALSE,
  alternative = "two.sided")
```

Output

```
> wilcox.test(change12 ~ P_tox,
+             data = Susbset_P_2_PD1pos_of_CD4mem_wide,
+             paired = FALSE,
+             alternative = "two.sided")

        Wilcoxon rank sum exact test

data:  change12 by P_tox
W = 99, p-value = 0.5621
alternative hypothesis: true location shift is not equal to 0

> wilcox.test(change13 ~ P_tox,
+             data = Susbset_P_2_PD1pos_of_CD4mem_wide,
+             paired = FALSE,
+             alternative = "two.sided")

        Wilcoxon rank sum test with continuity correction

data:  change13 by P_tox
W = 157.5, p-value = 0.5112
alternative hypothesis: true location shift is not equal to 0
```

```
> wilcox.test(change23 ~ P_tox,
+             data = Susbset_P_2_PD1pos_of_CD4mem_wide,
+             paired = FALSE,
+             alternative = "two.sided")

        Wilcoxon rank sum test with continuity correction

data:  change23 by P_tox
W = 101.5, p-value = 0.4834
alternative hypothesis: true location shift is not equal to 0
```

No significant differences

Mixed ANOVA, Repeated measures ANOVA, Wilcoxon-rank test

Panel 2 Toxicity VS no Toxicity on multiple timepoints

P_2_PD1pos_of_CD8 (PD1+ fraction of CD8+ T cells)

#Mixed ANOVA

```
> mixed_anova_P_2_PD1pos_of_CD8 <- aov_ez(id = "P_UNI",
                                          dv = "P_2_PD1pos_of_CD8",
                                          data = subset(Panel2_possiblevals, c(P_tox != "HD" & P_time != 2)),
                                          between= "P_tox",
                                          within = "P_time",
```

Output

```
Anova Table (Type 3 tests)

Response: P_2_PD1pos_of_CD8
       Effect    df  MSE       F  ges p.value
1       P_tox 1, 36 0.02    0.70 .015    .407
2      P_time 1, 36 0.01 7.49 ** .045    .010
3 P_tox:P_time 1, 36 0.01  4.08 + .025    .051
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘+’ 0.1 ‘ ’ 1
```

#Repeated-measures anova to check in which group the significant effect for time is

```
> Repeated_measures <- subset(Panel2_possiblevals, c(P_tox != "HD" & P_time != 2))
  %>%
group_by(P_tox) %>%
anova_test(dv = P_2_PD1pos_of_CD8, wid = P_UNI, within = P_time) %>%
get_anova_table() %>%
adjust_pvalue(method = "bonferroni")
```

```
> Repeated_measures
```

Output

```
# A tibble: 2 × 9
  P_tox       Effect   DFn   DFd     F     p `p<.05`   ges p.adj
  <fct>       <chr>  <dbl> <dbl> <dbl> <dbl> <chr>   <dbl> <dbl>
1 No toxicity P_time     1    18 0.369 0.551 ""      0.003 1
2 Toxicity    P_time     1    18 8.66  0.009 "*"     0.149 0.018
```

#Effect is in toxicity in time to wilcox test to see where the difference is

```
> Susbset_P_2_PD1pos_of_CD8 <- subset(Panel2_possiblevals,c(P_tox != "HD")) %>%
  select(P_2_PD1pos_of_CD8, P_tox, P_time, P_UNI)
```

Subset wide instead of long

```
> Susbset_P_2_PD1pos_of_CD8_wide <-Susbset_P_2_PD1pos_of_CD8 %>%
  pivot_wider(names_from = "P_time", names_prefix= "CD8pos_",
                                    values_from = P_2_PD1pos_of_CD8, values_fill = NA)
```

#Calculate change scores

```
> Susbset_P_2_PD1pos_of_CD8_wide$change12 <-
  Susbset_P_2_PD1pos_of_CD8_wide$CD8pos_2-
  Susbset_P_2_PD1pos_of_CD8_wide$CD8pos_1

> Susbset_P_2_PD1pos_of_CD8_wide$change13 <-
  Susbset_P_2_PD1pos_of_CD8_wide$CD8pos_3-
  Susbset_P_2_PD1pos_of_CD8_wide$CD8pos_1

> Susbset_P_2_PD1pos_of_CD8_wide$change23 <-
  Susbset_P_2_PD1pos_of_CD8_wide$CD8pos_3-
  Susbset_P_2_PD1pos_of_CD8_wide$CD8pos_2
```

Wilcoxon-rank test

```
> wilcox.test(change12 ~ P_tox,
  data = Susbset_P_2_PD1pos_of_CD8_wide,
  paired = FALSE,
  alternative = "two.sided")
```

Output

```
        Wilcoxon rank sum exact test

data:  change12 by P_tox
W = 63, p-value = 0.0392
alternative hypothesis: true location shift is not equal to 0
```

```
> wilcox.test(change13 ~ P_tox,
  data = Susbset_P_2_PD1pos_of_CD8_wide,
  paired = FALSE,
  alternative = "two.sided")
```

Output

```
        Wilcoxon rank sum exact test

data:  change13 by P_tox
W = 104, p-value = 0.02527
alternative hypothesis: true location shift is not equal to 0
```

Significant differences were found between baseline and on-treatment samples for PD-1 expression in CD8+ T cells between patients with and without toxicity. This was left out of the results because differences in PD-1 staining and gating in FlowJo might have caused discrepancies between baseline and on-treatment values, therefore we cannot assure this result to be reliable.

Wilcoxon-rank test at one timepoint

Panel 2 Response and toxicity

Wilcoxon-rank test between groups with Response_Tox variable ('Healthy donor', 'Non-responders with toxicity', 'Non-responders without toxicity', 'Responders with toxicity' and 'Responders without toxicity') for CD4+/CD8+ T cell ratio at baseline

#subset with Response_Tox variable with CD4+/CD8+ T cell ratio at baseline

```
> subset_responder_tox_BASE <- Panel2_possiblevals %>% select(P_UNI,
  Response_Tox, ratio_CD4_CD8, P_time)

> subset_responder_tox_BASE$P_time[subset_responder_tox_BASE$P_time == 2] <-
  NA
> subset_responder_tox_BASE$P_time[subset_responder_tox_BASE$P_time == 3] <-
  NA

> subset_responder_tox_BASE <- na.omit(subset_responder_tox_BASE)
```

#Perform Wilcoxon-rank test

```
> WIL_cox_ratioCD4_CD8_response_tox <- wilcox_test(ratio_CD4_CD8 ~ Response_Tox,
  data = subset_responder_tox_BASE)
```

Output

```
> WIL_cox_ratioCD4_CD8_response_tox
# A tibble: 10 × 9
   .y.          group1                      group2                    n1    n2 statistic     p p.adj p.adj.signif
 * <chr>        <chr>                       <chr>                  <int> <int>     <dbl> <dbl> <dbl> <chr>
 1 ratio_CD4_CD8 HD                          Non-responders with toxi…     5    10        32 0.44      1 ns
 2 ratio_CD4_CD8 HD                          Non-responders without t…     5     8        21 0.943     1 ns
 3 ratio_CD4_CD8 HD                          Responders with toxicity      5     6        16 0.931     1 ns
 4 ratio_CD4_CD8 HD                          Responders without toxic…     5     6        12 0.662     1 ns
 5 ratio_CD4_CD8 Non-responders with toxicity Non-responders without t…    10     8        36 0.762     1 ns
 6 ratio_CD4_CD8 Non-responders with toxicity Responders with toxicity     10     6        25 0.635     1 ns
 7 ratio_CD4_CD8 Non-responders with toxicity Responders without toxic…    10     6        20 0.313     1 ns
 8 ratio_CD4_CD8 Non-responders without toxicity Responders with toxicity   8     6        23 0.95      1 ns
 9 ratio_CD4_CD8 Non-responders without toxicity Responders without toxic…  8     6        17 0.414     1 ns
10 ratio_CD4_CD8 Responders with toxicity    Responders without toxic…     6     6        13 0.485     1 ns
```

No significant difference between groups at baseline.
This analysis was performed at all timepoints but no significant differences were found.