DETERMINATION OF INTRACELLULAR REACTIVE OXYGEN SPECIES IN T-CELL SUBSETS OF HIV+ PATIENTS ON CONTINUOUS cART

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ABSTRACT

Background: Reactive oxygen species (ROS) are generated at physiological levels as a result of cellular metabolism and contribute to cellular interaction and immune response. Elevated ROS may cause cell stress, damage, and apoptosis, and have been detected in different pathological states of infectious and non-infectious etiology.

Aim: To evaluate the association between intracellular ROS in T-cell subsets and HIV VL in chronic HIV infection.

Material and methods: Whole blood samples (Li-heparin, n=33) were analyzed during routine immune monitoring in two groups of HIV+ patients: A (n=21), on continuous cART for at least 2y, with sustained viral suppression (HIV VL<40 copies/ml) and group B (n=12) on cART for less than 2y, average HIV VL 92330 c/ml. Percentage and absolute counts (AC) of CD4+ and CD8+T cells were determined by flow cytometry (Multitest, BD Trucount™ tubes, FACS Canto II). Fluorometric ROS assay kit (Sigma-Aldrich) was adapted for flow cytometry analysis to detect intracellular ROS in CD4+ and CD8+ T-cells (FACSDiva 6.1.2).

Results: The average CD4AC did not differ significantly between group A and B (714 vs. 568, p>0.05), unlike the CD4/CD8 ratio (1.2 vs. 0.6, p<0.01). The mean fluorescence intensity (MFI) of CD4+T intracellular ROS was significantly lower in group A (mean MFI 1744 vs. 2492, p<0.05), unlike the CD8+T cell ROS content (1753 vs. 2129, p>0.05). Noteworthy, CD4+T intracellular ROS correlated positively with HIV VL (R=0.5, p<0.05), unlike CD8+T ROS. On the other hand, positive correlations between CD8+T ROS and cART duration, as well as age (R=0.5, p<0.05 for both) were observed in group A.

Conclusions: CD4+T ROS production may be an indicator of residual HIV activity in the settings of undetectable HIV VL. The combined effects of ageing and long-term cART affect mostly the CD8+T cell compartment.

Key words: *HIV, ROS, immune recovery, antiretroviral therapy*

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INTRODUCTION:

The existence of free radicals and their essential role in biological systems was suggested for the first time in 1954 by Commoner et al. (1). The free radicals are small molecules, which exist independently and at the same time contain one or more unpaired electrons (2, 3), which is the reason for their strong reactivity and a wide range of intra- and intercellular interactions.

Reactive oxygen species (ROS) include free oxygen radical species such as the hydroxyl radical ($\cdot$OH), and superoxide anion ($O_2 \cdot -$) as well as non-radical forms and hydrogen peroxide (H_2O_2), which are less reactive (4, 5). The largest source of ROS is oxidative phosphorylation in mitochondria (6, 7). ROS have pleiotropic effects, modifying a wide range of cellular functions and signal-transduction pathways (6, 8) and contributing in particular to the innate and adaptive immune response: initiation of macrophages activation, antigen cross-presentation, regulation of T-cell activation and functions (5, 9, 10). On the other hand, the increased formation of various ROS or the reduction of cellular antioxidant capacity leads to „oxidative stress", which ultimately causes apoptosis. This phenomenon is observed in various pathologies with infectious and non-infectious etiology, such as carcinogenesis (11,

12), neurodegeneration (13, 14), atherosclerosis, diabetes (15, 16) and aging (4, 17) or due to exogenous factors including ultraviolet and gamma radiation, smoke and other air pollutants, as well as several drugs and chemicals (5).

The role of oxidative stress in HIV pathogenesis has been addressed by numerous studies with somewhat inconsistent results. Reduced antioxidant capacity due to depletion of glutathione (GSH) in plasma, lymphocytes, monocytes and lung epithelial cells was described in HIV-infection. Thioredoxin (TRX) was found depleted in lymph node dendritic cells but elevated in the plasma of HIV-infected patients; reduced superoxide dismutase (SOD) levels in plasma and monocytes and H_2O_2 production in monocytes were associated with high HIV viral load (7). Indicators of elevated oxidative stress were detected in neutrophils, monocytes and astrocytes (18) in relation to TGF-β activation and induction of regulatory T cells (Foxp3+CD4+CD25+) (19).

It is well known that ROS production is induced by HIV proteins located on the viral envelope, such as transactivator of transcription (Tat), viral protein r (Vpr), negative regulatory factor (Nef) and glycoprotein 120 (gp120) (3, 20, 21, 22, 23). Nevertheless, ROS accumulation at the level of CD4+ and CD8+T cell subsets at different stages of HIV infection and during long-term combination antiretroviral therapy (cART) is poorly investigated. Contemporary cART is able to induce sustained viral suppression and immune restoration, though without complete viral elimination, bringing forward the problems of latent HIV reservoirs, low-level immune activation and accelerated ageing (24, 25, 26). ROS may increase HIV replication by reactivating LTR (long terminal repeats) in the latently infected cells through NF-κB –dependent mechanism (7). Clarifying the possible role of ROS for the activation of latent HIV in cART+ patients and understanding the mechanisms and the pathways that HIV uses to generate oxidative stress are of great importance for the successful monitoring and personalization of cART in long-term treated patients (10, 18).

Our current study aims to evaluate intracellular ROS in CD4+ and CD8+ T-cell subsets in relation to HIV VL and the residual immune activation in HIV+ patients on successful cART.

MATERIAL AND METHODS
Study design and participants
Peripheral blood samples (Lithium heparin, n=33) were obtained during routine immune monitoring of HIV+ patients registered at the Specialized Hospital for Active Treatment of Infectious and Parasitic Diseases, Sofia. Two groups were defined to evaluate the early and long-term effects of cART: Group A, patients on continuous cART for a minimum of two years, with undetectable viral load, and group B, treated less than two years, respectively **(Table 1)**.

Viral load determination
HIV viral load was determined in plasma by reverse transcription polymerase chain reaction (Abbott Real-Time HIV-1), over the range of 40 to 10,000,000 copies/ml.

Cell isolation and flow cytometric analysis:
The percentage and absolute counts (AC) of CD4+ and CD8+T lymphocytes were determined by direct multiparameter flow cytometry (Multitest CD3/CD8/CD45/CD4/TRUCount, FACS Canto II, BD Biosciences), **(Fig.1A)**.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque Plus (Sigma-Aldrich). For determination of intracellular ROS in living T-cells, a cell-permeable green fluorescent sensor (λex = 490/λem = 520 nm, Sigma-Aldrich) was used, forming with intracellular ROS a fluorometric complex proportional to their quantity. Briefly, freshly isolated $5x10^4$ PBMCs were incubated for 60 min at 37°C with ROS sensor, stained with CD3APC/CD4-CF-Blue/CD8-PerCP and analysed by flow cytometry (FACSDiva 6.1.2). The same cells, not incubated with the sensor were used as negative control. ROS levels were characterized by the mean fluorescence intensity (MFI_{ROS}) of the stained sample in the FITC-channel after subtracting the background fluorescence of the negative control **(Fig. 1B)**.

Statistical analysis:
Parametric data are presented as mean ± SD. Comparisons between groups were performed with one-tail unpaired T-test, and relationships between two variables were analyzed by Spearman's rank correlation coefficient. P values less than 0.05, at CI 0.95 were considered significant (SPSS Statistics v.21.0). Graphpad Prism v.9.0 was used for the graphical representation of the results.

RESULTS AND DISCUSSION:
The demographic and basic laboratory characteristics of the study groups are given in

Table 1 Demographic, clinical and laboratory characteristics of study participants. All data is represented as mean±SD.

| HIV+ participants	Group A	Group B	Unpaired T-test
Number (n)	21	13	N/A
Age (years)	43 ±8	33 ±6	p<0.001***
Time after diagnosis of HIV+ (years)	10.7 ±7.6	5.8 ±5.1	p<0.05*
Time between diagnosis and start of cART (months)	15.9±39.5	12.6±25.9	p>0.05
cART duration (months)	103 ±73	13 ±9	p<0.001***
Viral load (copies/ml)	35.8 ±9.0	92330 ±2390	p>0.05
Baseline* CD4 AC (cells/µl)	358 ±243	394 ±360	p>0.05
Baseline CD4/CD8 (ratio)	0.54 ±0.59	0.53 ±0.54	p>0.05
Last CD4 AC (cells/µl)	714 ±313	568 ±383	p>0.05
Last CD4/CD8 (ratio)	1.16 ±0.80	0.61 ±0.42	p<0.01**

*At start of cART
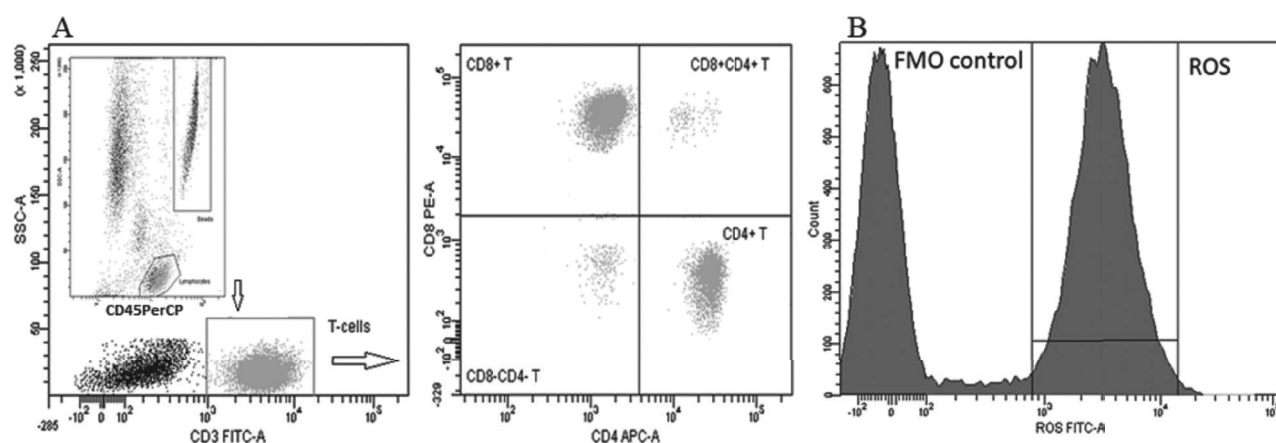
Fig.1 Flow cytometry analysis of ROS in T cells. (A) Gating strategy for CD4+ and CD8+ T-cell subsets. Lymphocytes were initially gated on side scatter properties and CD45 expression (upper left panel), T cells were then gated on the expression of CD3 (left panel), and further subdivided into CD4+ and CD8+ T cells (middle panel). **(B) Determination of intracellular ROS in CD8+ and CD4+ T cells using a ROS sensor.** MFI was measured in the FITC-channel. A Fluorescence Minus One (FMO) control was used to set markers for the ROS-positive population.

Table 1. As expected, long term-treated patients (group A) differed significantly in age, presumed disease duration (time since HIV diagnosis), and time on cART as compared to group B. Importantly, baseline CD4 AC and CD4/CD8 in group A and B (358±242 vs. 394±360 and 0.54±0.59 vs. 0.53±0.54), as well as the time interval between diagnosis and the start of cART (15.9±39.5 vs. 12.6± 25.9) were similar (p>0.05 for all comparisons) giving the grounds to compare the effect of cART with different duration. There were no significant differences between the groups regarding HIV VL, though in four patients from group B the virus was still detectable. Moreover, the last measured CD4 AC were similar (714±313 vs. 568±384, p>0.05). The only parameter which differentiated between the groups was a higher mean CD4/CD8 ratio in long-term treated patients (A) (1.16±0.8 vs. 0.61±0.42, p<0.05). In fact, 67% of patients who had recently started ART (B), and 41% of those on long-term treatment (group A) had suboptimal CD4/CD8 ratio (<0.9).

The mean fluorescence intensity of CD4+T ROS (MFI_{ROS}) in group A was significantly lower as compared to group B: (mean ±SD) 1744± 396 vs. 2492±1239, p<0.05. At the same time, CD8+ T MFI_{ROS} in group A was also lower than in group B (1753±542 vs. 2129±989, respectively), but the difference did not reach statistical significance (p>0.05) (**Fig. 2**).
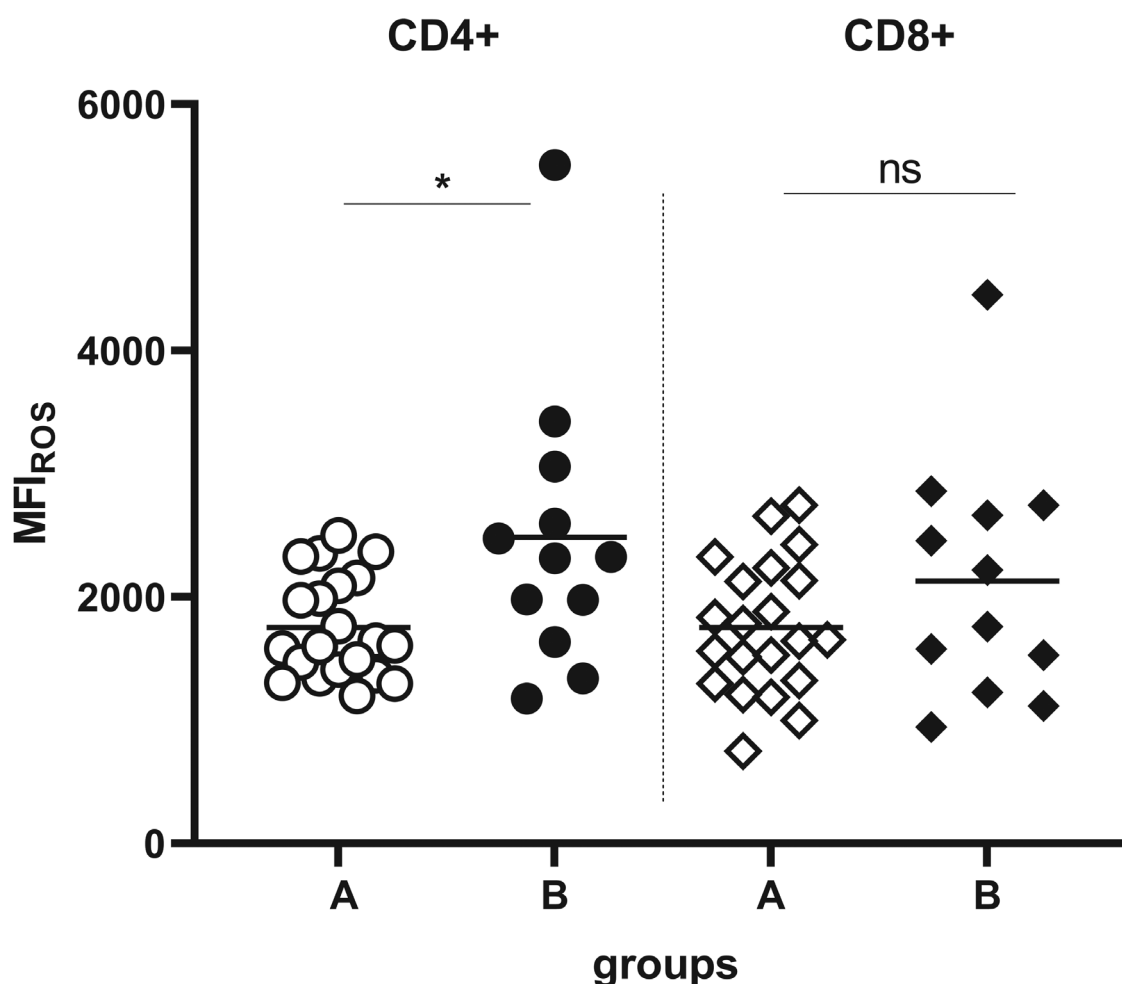

Fig. 2 Comparison of MFI_{ROS} in CD4+ and CD8+ T cells between groups A and B. The difference was statistically significant for CD4+T cells p<0.05 (*), unlike CD8+ T cells, (p>0.05).

Noteworthy, CD4+T MFI_{ROS} correlated with HIV VL (R=0.5, p<0.05) unlike CD8+T MFI_{ROS} (**Fig. 3A**). In group A, we found a positive correlation between CD8+T MFI_{ROS} and age, as well as cART duration (R=0.5, p<0.05 for both) (**Fig. 3C, D**). Finally, CD4/CD8 ratio in group A correlated negatively with CD4+T MFI_{ROS} (p<0.05, R=-0.47) (**Fig. 3B**). Contemporary cART suppresses plasma HIV RNA below detection limit of laboratory assays and restores CD4 T cell pool of infected patients by preventing new rounds of productive infection, depending on its baseline state. Usually, most HIV+ individuals achieve viral suppression (less than 50 copies/ml) in a couple of months after initiation of cART (27). According to accepted criteria, both studied groups demonstrated a virological and immunological response to cART. The quick restoration of CD4 AC was probably associated with the comparatively high baseline CD4AC in both groups, as observed by others as well (28). However, a number of studies have shown that neither HIV VL, nor CD4 AC are exhaustive correlates of immune restoration. The subset ratio CD4/CD8 is a more sensitive marker of ongoing low-level immune activation of variable origin in the settings of cART, involving mostly the CD8 T cell compartment and ultimately leading to its exhaustion (29). In our hands, 67% of patients who had recently started cART, and – remarkably, 41% of those on long-term treatment had a pathologically decreased CD4/CD8 ratio (<0.9) that
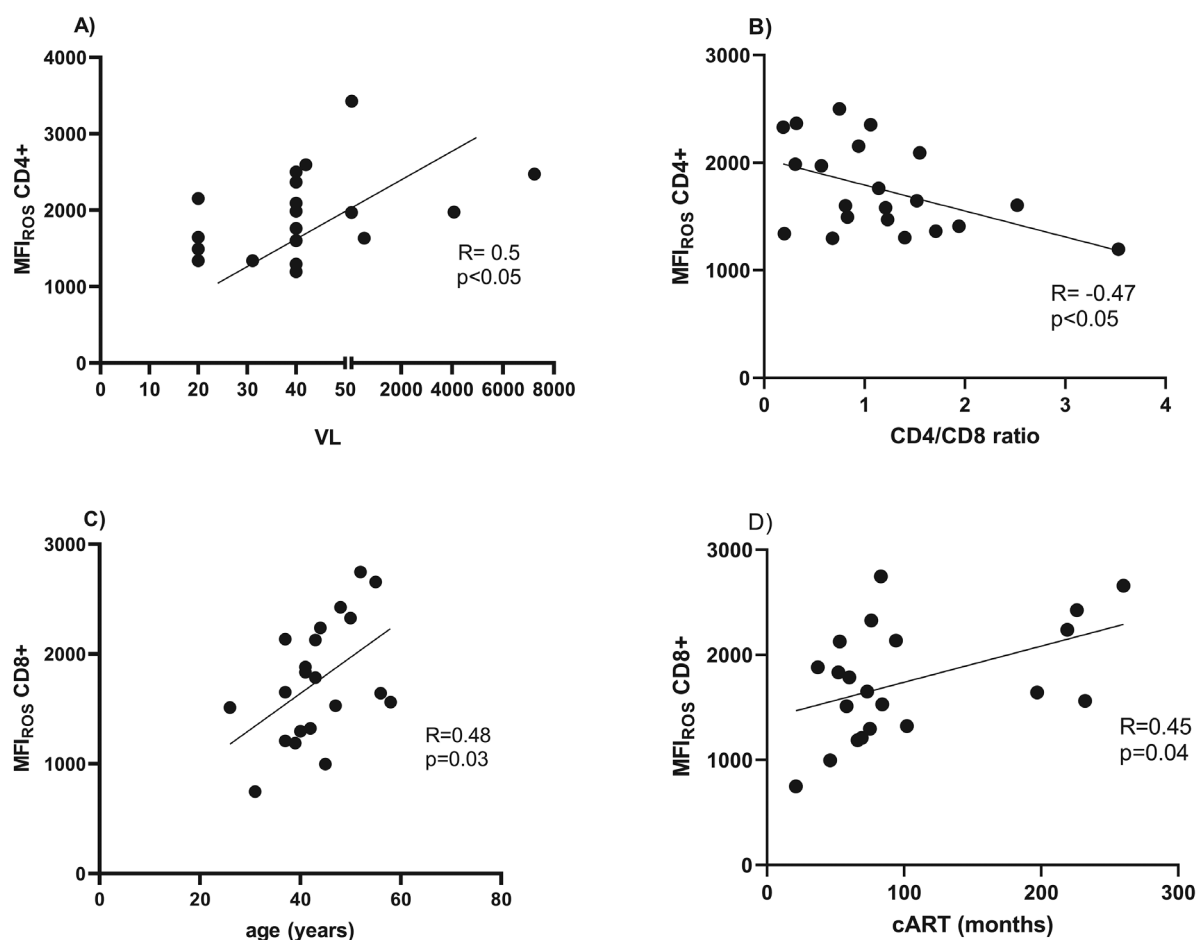
Fig. 3 Correlations of intracellular ROS: CD4+MFI$_{ROS}$ correlated positively with viral load **(A)**, and inversely - with CD4/CD8 ratio **(B)**; Intracellular CD8+T MFI$_{ROS}$ correlated positively with age **(C)**, as well as with cART duration **(D)**.

could reflect any immune activation, including one caused by the slowly replicating virus and affecting CD4 T cells, or one due to opportunistic infections, involving mostly the CD8 T cell compartment. In this aspect, it was interesting whether ROS content of CD4 and CD8 T cells could clarify this issue.

According to our results, it was CD4+T ROS content that differentiated between group A and B, and correlated with HIV VL. Moreover, within the group with undetectable HIV VL (A), CD4 T ROS correlated nicely and inversely with CD4/CD8 ratio, suggesting that the low level immune activation in patients with suboptimal ratio might actually reflect reactivation of latent HIV reservoirs. On the other hand, CD8 T cell ROS while not significantly differentiating between groups A and B, correlated directly with age and cART duration.

Oxidative stress has been implicated in many aspects of HIV pathogenesis, such as increased viral replication, CD4+ T-cell damage, altered immune response, and antiretroviral drug toxicity (3, 23). Most of the conflicting evidences on oxidative stress in HIV infection were reported in conditions without cART (20). A few studies reported on ROS levels in CD4 and CD8 T cell subsets of HIV+cART-treated patients. Yu et al. found that ROS accumulation in CD4+T cells was increased in HIV+ patients as compared to HIV- control subjects, while HIV infection seemed to have no effect on ROS accumulation in CD8+T cells. Further on, they did not observe any difference between CD4+ T ROS levels after 0.5-1- 2 and 3 years of cART. Interestingly though, the introduction of cART led to the abnormal accumulation of ROS in CD8+T cells as compared to healthy controls. In fact, these results are in line with ours, strengthening the idea that HIV infection mainly influences ROS accumulation in CD4+T cells, while long-term ART affects mostly the CD8 compartment (30). Unlike Yu et al. we demonstrate that long term cART significantly reduces CD4 ROS levels. Upon this background a slight increase of CD4+MFI ROS may be a sensitive early sign of HIV reactivation.

HIV replicates in highly oxidized environment. CD4+ cells shift from their resting state into an active state of immune response via a cascade of internal oxidative reactions, which stimulate HIV genes to reproduce in infected cells, while the increased metabolism of these cells provides the cellular factors that are useful for production of new viral particles (31).

The association between CD8+ ROS content and cART duration, as well as the age of patients from group A is also in line with the observations of others. Functionally exhausted CD8+ T cells in the settings of some cancers and chronic infections were shown to have signs of a broadly dysregulated metabolic state and evidence of oxidative stress as measured by the accumulation of ROS. Also, cART suppressed HIV+ patients tend to have a higher ROS content across most CD8+ T cell subsets, either related to the effects of residual infection and/or the impact of antiretroviral therapy on T cell metabolism (32). Unlike CD4+ ROS, and in line with our results, ROS content of HIV-specific CD8+ T cells in the study of Deguit et al. did not vary according to the extent or mechanism of viral control (32).

Our study has some limitations. First of all, participants in the two groups were not age-matched. This might have influenced the difference between the amount of oxygen radicals in group A (older) and group B, having in mind that ROS significantly increase with age. Second, the small number of participants and the absence of control HIV- volunteers as well as ART-naïve HIV+ patients have also limited the interpretation of the severity of oxidative stress.

CONCLUSIONS:

Our data suggest that elevated CD4+ ROS level in virally suppressed long-term treated HIV+ patients can be an indicator of residual viral activity. On the other hand, the combined effects of aging and long-term therapy predominantly affect CD8+ T cells and their ROS content might serve as an indicator of advancing functional exhaustion.

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