Stable IL-2 Decision Making by Endogenous c-Fos Amounts in Peripheral Memory T-helper Cells*

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The cytokine IL-2 performs opposite functions supporting efficient immune responses and playing a key role in peripheral tolerance. Therefore, precise fine-tuning of IL-2 expression is crucial for adjusting the immune response. Combining transcription factor analysis at the single cell and the single nucleus level using flow cytometry with statistical analysis, we showed that physiological differences in the expression levels of c-Fos and NFATc2, but not of c-Jun and NF-κBp65, are limiting for IL-2 decision making.

Results: Endogenous expression levels of c-Fos and NFATc2, but not of c-Jun and NF-κBp65, are limiting for IL-2 decision making. Combining transcription factor expression analysis at the single cell and the single nucleus level using flow cytometry with statistical analysis, we showed that physiological differences in the expression levels of c-Fos and NFATc2, but not of c-Jun and NF-κBp65, are limiting for the decision whether IL-2 is expressed in a strongly activated human memory T-helper (Th) cell. Variation in the expression of c-Fos leads to substantial diversity of IL-2 expression in ~40% of the memory Th cells. The remaining cells exhibit an equally high c-Fos expression level, thereby ensuring robustness in IL-2 response within the population. These findings reveal how memory Th cells benefit from regulated variation in transcription factor expression to achieve a certain stability and variability of cytokine expression in a controlled manner.

The main transcription factors regulating IL-2 expression following T cell stimulation are NFATc2, NF-κB (p65/p50), AP-1 (c-Fos and c-Jun), and OCT (8–11). It has been shown using reporter gene assays and point mutation approaches in cell lines that each of these transcription factors is crucial and that they act together to induce IL-2 production (8–13). However, neither c-Fos nor NFATc2 nor NF-κBp65 single knock-out mice exhibited an impaired IL-2 expression (14–16), whereas c-Fos transgenic mice exhibit a five times higher IL-2 expression compared with wild type mice (17). These discrepancies might be due to compensatory effects by other members of the Fos and NFAT families (18). Transgenic mouse lines containing a GFP reporter gene controlled by the 8.4-kb promoter region of the murine IL-2 gene were used to study the IL-2 expression quantitatively (19). However, these mice exhibited divergent abilities to express the transgene compared with the endogenous IL-2 gene, possibly because of restricted potential regulatory sequences in transgenes (19). Data from the published transcription factor knock-out and transgenic mice are summarized in supplemental Table S1.

Thus, neither reporter gene assays nor mice (knock-out or transgenic) are sufficient alone for studying quantitative aspects of endogenous IL-2 expression. To address the question of how physiological quantitative differences in transcription factor expression are translated into qualitative differences in IL-2 expression in peripheral human Th cells, we used quantitative determination of transcription factor expression and cytokine expression at the single cell level combined with statistical analysis. We measured physiological protein expression levels within Th cell populations from healthy individuals to study the quantitative impact of NFATc2, c-Fos, c-Jun, and NF-κBp65 on IL-2 decision making in primary human Th cells. According to our findings, when human memory Th cells are subjected to strong stimulation the levels of the transcription factors NFATc2 and c-Fos, but not their activation and not c-Jun and NF-κBp65, correlate with IL-2 decision making at the single cell level. Moreover, variation in c-Fos expression of the cells generates substantial diversity and robustness of IL-2 expression within the memory Th cell population in human...
with Feinerman et al. (20) our data show that the used quantitative single cell approach is useful for uncovering basic mechanisms during gene regulation, such as the existence and robustness of threshold levels, as well as the contribution of endogenous protein levels to cell response within a cell population.

**EXPERIMENTAL PROCEDURES**

**Human T Cell Isolation and Stimulation**—Peripheral blood mononuclear cells from healthy volunteers were prepared using Ficoll PAQUE gradients from leukocyte concentrates obtained from the blood bank of the Red Cross. Positively selected CD4+ cells were further depleted of CD45RA+ Th cells to a purity of >97% CD4+CD45RO+ memory Th cells (MACS Separation Reagents; Miltenyi Biotech). Cells were cultured in RPMI supplemented with 10% fetal calf serum and stimulated either with 10 ng/ml PMA and 1 µg/ml ionomycin or anti-CD3/CD28 antibody-coupled beads (25 µl/1 × 10^6 cells; Dynal Beads, Invitrogen). The MEK1/2 inhibitor U0126 (Biomol GmbH) was preincubated with Th cells for 20 min before stimulation and was used in concentrations of 0.01–250 µM.

**Isolation of Intact Nuclei and Flow Cytometric Analysis**—Nuclei were isolated and analyzed as previously described (21). Shortly, isolated nuclei were fixed/permeabilized using the FOX3 staining buffer set (ebioscience) and stained with FITC-coupled anti-c-Fos, anti-NF-κB (Santa Cruz Biotechnology), or anti-NFATC2 antibodies (BD) and allophycocyanin c-coupled anti-FOX3 antibody (ebioscience). In addition, the nuclei were stained with 1 µg/ml propidium iodide prior to flow cytometric analysis. The nuclei were gated according to forward and side scatter and propidium iodide staining, and doublletes were excluded by pulse processing. FlowJo Software was used for analysis.

**Data Transformation, Normalization, and Partitioning**—The free software package R (bioconductor) was used for data transformation, normalization, and partitioning. Transformation was performed to linearize the data using either the log or the asinh function. Normalization was performed by subtracting the mean fluorescence intensity (MFI) from the fluorescence intensity (FI) of each cell. Partitioning into 4 (see Fig. 4) or 11 (see Fig. 5) bins was performed according to the FI of a transcription factor per cell. The number of IL-2-producing cells was determined for each bin. Heat maps (see Figs. 6 and 7) were generated according to the FI of c-Fos, NFATC2, and IL-2 expression levels.

**Statistical Analysis and Mathematical Modeling**—To model the dependence of the frequency of IL-2-producing cells from the c-Fos and NFATC2 level, we used the following models: 1) a mass action model (linear model), 2) a Michaelis-Menten model, and 3) a Hill model. The models increase in complexity, as is apparent from the numbers of parameters.

\[
\text{% IL-2} = \frac{a \cdot TF}{c + TF} + b \quad (\text{Eq. 1})
\]

The parameters of each model were estimated by minimizing the distance from the simulated data to the experimental data (least square fitting). To compare the models, we used the Akaike information criterion with correction for finite sample sizes (AICc; Equation 4), which takes into account the distance between models, as well as considering the complexity of the model. With increasing complexity of a model (increasing numbers of parameters), it is easier to explain the data but more difficult to predict the data (overfitting).

\[
\text{AICc} = n \times \ln(n) + 2k + \frac{2k(k + 1)}{n - k - 1} \quad (\text{Eq. 4})
\]

\[
\text{AICc} = n \times \ln\left(\frac{\text{RSS}}{n}\right) + 2k + \frac{2k(k + 1)}{n - k - 1} \quad (\text{Eq. 5})
\]

where \(k\) is the number of parameters, \(n\) is the number of data points, and RSS is the residual sum of squares. To model possible interaction of c-Fos and NFATc2, we used the best fitting model from above and used an additive (Equation 6) and a multiplicative (Equation 7) approach to describe the heat map data on single cell level containing c-Fos, NFATc2, and IL-2 expression levels.

\[
\text{% IL-2} = a_1 \cdot c_{-fos} + a_2 \cdot NFATc2^{d_1} \quad (\text{Eq. 6})
\]

\[
\text{% IL-2} = a_1 \cdot c_{-fos} + a_2 \cdot NFATc2^{d_2} \quad (\text{Eq. 7})
\]

The multiplicative model (Equation 7) describes the independent DNA binding of each transcription factor as shown by Bintu et al. (22). In contrast, the additive model (Equation 6) describes the cooperative behavior of DNA binding of both transcription factors (22). We adjusted the model parameters \(a_1\) and \(b\) (expansion and offset) to the additional data and compared the two models with AICc.

**RESULTS**

**Stable IL-2 Expression in Peripheral Memory Th Cell Population of Individual Donors**—In view of the fact that IL-2 plays a crucial role for both immunity and peripheral tolerance, we assume that healthy individuals have a tailored and stable IL-2 production. For measurements, we chose the peripheral memory Th cell population, because it is the largest cell population in the periphery able to produce IL-2.

After stimulation, the relative amount of IL-2-producing cells is strongly up-regulated, leading to a 4-fold increase from 2 to 5 h after stimulation (Fig. 1). For each of the five individual donors, the relative amount of IL-2-producing cells (after 2 h as well as after 5 h) is carefully regulated, because variations over four measurements within 6 weeks are small. These results sug-
c-Fos Limits IL-2 Decision Making

FIGURE 1. Stability of IL-2 production of individual donors after Th cell stimulation. Isolated CD4+CD45RA- Th cells from five donors were collected on four different days within 6 weeks. The cells were stimulated with PMA/ionomycin for 2 or 5 h and analyzed for IL-2 expression by flow cytometry. The frequency of IL-2 producers and the standard deviation of mean values are depicted.

Diminished Transcription Factor Expression in FOXP3+ Compared with FOXP3− Memory Th Cells—To determine the impact of physiological amounts of transcription factors on IL-2 decision making, we used ex vivo isolated memory Th cells (CD4+CD45RO+CD45R-CD45RA-) from human peripheral blood and measured the expression of NFATc2, NF-κBp65, c-Fos, and c-Jun at the single cell level by flow cytometry.

First we compared the transcription factor levels of Foxp3− with Foxp3+ Th cells (Fig. 2A), because the latter are widely known to be incapable of producing IL-2 and other effector cytokines (23). Foxp3+ Th cells express lower amounts of NFATc2, c-Jun, and NF-κBp65 than Foxp3− Th cells in unstimulated ex vivo isolated memory Th cells (Fig. 2A). Furthermore, the stimulation-dependent up-regulation of c-Fos is higher in FOXP3+ than in FOXP3− Th cells. All of these data were confirmed by Western blot analysis of MACS-sorted memory Th cells that were subsequently FACs-sorted into non-Treg (CD127+CD25−FOXP3−) and Treg cells (CD127loCD25+FOXP3+) (purity of FOXP3+ cells > 96%). Accordingly, small differences in the MFI values of the four studied transcription factors measured by flow cytometry substantially represent visible differences in protein amounts detected by Western blotting (Fig. 2A).

IL-2 Producing Th Cells Express Higher Amounts of NFATc2 and c-Fos, but Not NF-κBp65 and p-c-Jun, than IL-2 Nonproducing Th Cells—To investigate whether the expression of NFATc2, NF-κBp65, c-Fos, and c-Jun differs between IL-2+ Th and IL-2− Th cells, we established co-stainings for IL-2 and the respective transcription factors. This does not only allow the correlation of the transcription factor expression directly with IL-2 decision making but also enables the relative quantification of all parameters at the single cell level using flow cytometry. We examined whether the low transcription factor expression observed in Treg cells is a general feature of IL-2 nonproducing, as compared with IL-2 producing, memory Th cells.

FOXP3− cells (85–95%) were gated according to their IL-2 expression using unstimulated samples as controls (Fig. 2B, top row). IL-2+ memory Th cells exhibited significantly higher NFATc2 (p = 0.0083) and c-Fos (p = 0.0082) expression than IL-2− Th cells in all of the donors studied (n = 7) (Fig. 2B). By contrast, NF-κBp65 (p = 0.9988) and p-c-Jun (p = 0.2007) were not differentially expressed (Fig. 2B). Similar results concerning the transcription factor expression in IL-2 producing and non-producing Th cells were obtained after stimulation with anti-CD3/CD28 antibodies (data not shown).

All Studied Transcription Factors Are Activated, Both in IL-2+ and IL-2− Memory Th Cells—Next we examined whether the activation of NFATc2, NF-κBp65, c-Fos, and c-Jun following strong stimulation of the cells is impaired in the IL-2−, by comparison with the IL-2+, memory Th or Treg cell population. Thereby, we exploited the fact that these transcription factors translocate from the cytosol into the nucleus following stimulation-dependent activation. We isolated nuclei from unstimulated and stimulated memory Th cells and stained them for NFATc2, c-Fos, NF-κBp65, and Foxp3, as previously described (21).

NFATc2 is activated in the whole memory Th cell population both in the Foxp3− and the Foxp3+ subpopulation following strong stimulation with anti-CD3/CD28 antibodies (Fig. 3A and data not shown) or PMA/ionomycin (Fig. 3B), because the MFI values of the whole population shifted compared with unstimulated control samples (n = 6). The complete memory Th cell population also activates c-Fos (n = 3) as well as NF-κBp65 (n = 3). Accordingly, p-c-Jun exhibited an equal phosphorylation and thus activation of c-Jun in both IL-2+ and IL-2− Th cells in the memory Th and Treg cell population (Fig. 3C). In summary, the lack of IL-2 expression in 10–50% of strongly stimulated memory Th cells is not due to variations in the activation of the transcription factors NFATc2, NF-κBp65, c-Fos, and c-Jun within the cell populations.

Even in Treg Cells, Activation of NFATc2, c-Fos, NF-κBp65, and c-Jun Is Not Impaired after Strong Stimulation—Several reports showed defects in the proximal TCR signaling cascade in FOXP3+ Th cells (24, 25). So far, it is not clear whether these defects have an impact on the activation of the main transcription factors and therefore play a decisive role for IL-2 expression in FOXP3+ Th cells. The transcription factor FOXP3 resides in the nucleus in unstimulated as well as in stimulated cells, and it was used to separate FOXP3+ nuclei from FOXP3− nuclei.

The activation of NFATc2, c-Fos, and NF-κBp65 is intact after anti-CD3/CD28 stimulation, as indicated by the shift in the MFI values of all nuclei compared with nuclei from unstimulated cells (Fig. 3B). The overlay of gated FOXP3− and FOXP3+ nuclei suggests that the activation of the transcription factors in Treg cells is comparable with FOXP3− memory Th cells. Consequently, under the conditions used, the differences in the proximal TCR signaling between FOXP3+ and FOXP3− Th cells do not affect the downstream transcription factor activation.

Quantification of Western blot experiments (Figs. 3C and 2A) revealed that both the amounts of c-Jun and the active p-c-Jun are lower in FOXP3+ than in FOXP3− Th cells. How-
However, the ratio of phosphorylated c-Jun to total c-Jun within each of the two Th cell populations was virtually equal (1.93 for FOXP3$^+$ and 2 for FOXP3$^-$ Th cells). It follows that the repression of IL-2 production in Treg cells is not due to impaired activation of NFATc2, c-Fos, c-Jun, and NF-$\kappa$Bp65.

The Amount of c-Fos and NFATc2 per Cell Is Limiting for IL-2 Decision Making in Memory Th Cells—Testing the hypothesis that physiological amounts of c-Fos and NFATc2 are limiting for IL-2 production in primary human memory Th cells usually requires overexpression or knockdown of these proteins. However, such manipulations of primary Th cells often lead to a different status quo not only of one transcription factor but also of the complete transcription factor network compared with unmodified ex vivo cells.

To circumvent these difficulties, we used a novel approach combining flow cytometry data analysis with statistical evaluation of single cell data. In the isolated memory Th cell populations of healthy donors, we correlated the physiological amounts

![Diagram](image-url)
FIGURE 3. Strong stimulation activates the transcription factors in all CD4⁺ CD45RO⁺ Th cells. A, sorted human CD4⁺ CD45RO⁺ Th cells were stimulated with anti-CD3/CD28 antibodies for 15 min (NFATc2 and NF-kBp65) or 2 h (c-Fos). Subsequently, isolated nuclei of unstimulated and stimulated Th cells were stained with propidium iodide (PI) and fluorophore-coupled antibodies for transcription factors. The nuclei were identified using a special gating strategy: first, according to their size and granularity; second, according to propidium iodide staining to exclude unlysed cells and cell debris; and third, according to the "doublette discrimination module" to exclude doublettes (FACSCalibur). Histogram overlays show transcription factor levels in isolated nuclei from unstimulated (gray line) and stimulated (black line) CD4⁺ CD45RO⁺ Th cells. The data of one representative donor of three (NFATc2) and six (c-Fos and NF-kBp65) are shown. B, histogram overlays show transcription factor expression in FOXP3⁺ and FOXP3⁻ gated nuclei from PMA/ionomycin stimulated and unstimulated CD4⁺ CD45RO⁺ Th cells (n = 3). C, immunoblot analysis shows relative amounts of c-Jun and p-c-Jun in flow cytometry-sorted FOXP3⁻ (CD127⁺⁺⁺CD25⁻⁻) and FOXP3⁺ (CD127⁺⁺⁺CD25⁺⁺) CD4⁺ CD45RO⁺ Th cells. Integrated intensities were determined using Lycor Odyssey® software (lower row) (n = 3). SSC, Side Scatter; FSC, Forward Scatter.
of NFATc2, NF-κBp65, c-Fos, and p-c-Jun with IL-2 production per cell. Specifically, the FI of the respective transcription factor, as a measure of the protein amount per cell, was divided into quartiles. Each quartile contained the same number of cells (Fig. 4A). Subsequently, the IL-2 expression in each quartile was plotted into histograms (Fig. 4B). Different amounts of c-Fos and NFATc2, but not of NF-κBp65 and p-c-Jun, exhibit a clear correlation with the number of IL-2-producing cells in FOXP3+ memory Th cells.

In contrast, partitioning the single cell data obtained from FOXP3+ Th cells showed that all transcription factors, NFATc2, NF-κBp65, c-Fos, and c-Jun, are limiting in Treg cells (data not shown). This is in agreement with the observed lower endogenous level of these transcription factors in Treg cells compared with memory Th cells.

Interestingly, the separate investigation of the IL-2 producing memory Th subpopulation revealed that, in a given healthy donor, the expression levels of c-Fos and NFATc2 define whether or not a single cell produces IL-2 rather than the amount of IL-2 per cell (supplemental Fig. S1). Expression of c-Fos and expression of c-Jun were up-regulated after strong T cell stimulation within 5 h, whereas the levels of NFATc2 and NF-κBp65 were quite stable (supplemental Fig. S2).

The Variability and Robustness of IL-2 Decision Making Is Mainly Due to Heterogeneity in Physiological c-Fos Expression Levels—To address the question of whether the range of the endogenous transcription factor expression levels can explain the IL-2 decision making in a single cell, we analyzed strongly stimulated memory Th cells from 9 healthy donors using flow cytometry. To compare and integrate data from different experiments and donors, we used the cytometer setup and tracking system of Becton Dickinson to produce consistent and reproducible data (supplemental Fig. S3). The flow cytometry data of each donor were normalized and transformed into a linear scale using the asinh function (Fig. 5A). Subsequently, the range of transcription factor expression level of each donor was divided into 11 equally spaced bins, and the frequencies of IL-2 producers were calculated for each bin.

The probability of a memory Th cell producing IL-2 under strong T cell stimulation conditions clearly depends on its levels of c-Fos and NFATc2 (Fig. 5B), but not on its levels of NF-κBp65 and c-Jun (supplemental Fig. S4). Stochastic variation in the expression of c-Fos gives rise to substantial diversity in IL-2 expression in memory Th cells (Fig. 5B, left panel). Approximately 40% of the memory Th cells have c-Fos expression levels that limit the probability of IL-2 production. High c-Fos expression levels in ~60% of the memory Th cells ensure the robustness of IL-2 expression within the memory Th cell population after strong T cell stimulation. A certain c-Fos level (in 6 of 11 bins) is sufficient to ensure maximum IL-2 response because higher c-Fos expression levels do not substantially increase the frequency of IL-2-producing cells. However, 10–15% of the memory Th cells are not able to produce IL-2 despite a high level of c-Fos per cell.

The variability in NFATc2 levels per cell has a lower impact on IL-2 decision making than c-Fos levels. High NFATc2 levels are able to increase the probability of IL-2 production by up to 20–30% (from 50 to 80% probability per bin), indicating that endogenous NFATc2 amounts enhances, but does not absolutely limit, IL-2 production under strong stimulation conditions (Fig. 5B, right panel). The highest variability in IL-2 production occurs in the central bins of NFATc2 expression (between −0.5 and 0.5 a.u.). On the whole, among the principal transcription factors studied, c-Fos determines the variability and robustness of IL-2 decision making under strong stimulation conditions.

Cooperative Interaction of c-Fos and NFATc2 in IL-2 Gene Expression—To gain a better understanding of the molecular mechanism underlying c-Fos- and NFATc2-dependent IL-2 production, we used three simple mathematical models: first, a straightforward mass action model describing a linear dependence of IL-2 production on the level of one transcription factor; second, a Michaelis-Menten model describing the dependence between the occupation of a single transcription factor DNA binding site and the concentration of the transcription factor (26); and finally, a Hill model describing the DNA binding of a transcription factor if there are two or more cooperative binding sites (27).

To discover the best fitting model, we took flow cytometry data from nine donors to estimate parameter values for different kinetics (Fig. 5C and supplemental Table S2) and compared the models using the goodness of fit, AICc, and the $\chi^2$ test (more details are provided under “Experimental Procedures”). Both the Michaelis-Menten equation and a Hill equation with the Hill coefficient $d = 1$ best describe the c-Fos-dependent IL-2 expression. Of the three tests of goodness performed, only...
the AICc method discriminates between the two models (supplemental Table S2). For NFATc2-dependent IL-2 expression, all three tests of goodness indicate that a Hill equation provides the best fit with the data (supplemental Table S2). We conclude that c-Fos acts as a transcription factor with a single binding site, whereas NFATc2 behaves as a transcription factor with at least two cooperative binding sites at the IL-2 promoter.

However, it has been established that c-Fos and NFATc2 are frequently associated at common AP-1/NFAT binding sites of genes after T cell stimulation. The proximal IL-2 promoter has four AP-1/NFAT and one NFAT binding sites (8–10, 28, 29). To determine whether c-Fos and NFATc2 indeed act cooperatively in IL-2 gene expression, we proposed and tested two models. The first model describes cooperativity between c-Fos and NFATc2 (additive model), whereas an alternative model describes the independent action of each transcription factor (multiplicative model) on IL-2 gene expression (22).

To evaluate both models, we established a three-parameter staining to quantify c-Fos, NFATc2, and IL-2 expression levels simultaneously at the single cell level by flow cytometry. The stimulated Th cells of three donors were measured. The range of the transcription factor expression levels was divided into two-dimensional bins, and the frequency of IL-2 producers was calculated for each bin containing more than two cells (Fig. 6A).

Certain combinations of c-Fos and NFATc2 expression levels do not occur in cells of the memory Th cell population, e.g. very low expression of one transcription factor and very high expression of the other. Furthermore, the plot shows that all of the

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**FIGURE 5. The probability of IL-2 production per cell depends on c-Fos and NFATc2 expression levels.** Sorted human CD4⁺CD45RO⁺ T cells were stimulated with PMA/ionomycin for 5 h and analyzed by flow cytometry. The data of different experiments and donors were integrated using the cytometer setup and tracking system of Becton Dickinson. A, flow cytometry data of nine donors were transformed into a linear scale using the asinh function and normalized to the mean FI of each transcription factor. Subsequently, the range of transcription factor expression level of each donor was divided into 11 bins. B, the frequency of IL-2 producers within each bin is depicted as a mean value of nine donors and the standard deviation of the mean. Background coloring indicates increasing levels of the transcription factor per cell within each bin. C, data were rescaled (lowest value is 0), and three models (linear kinetic, Michaelis-Menten kinetic and Hill kinetic) were fitted to the data. The model that fits best to the data is shown as a solid line.
memory Th cells expressing high levels of both transcription factors produce IL-2 (Fig. 6A, red). However, just a few cells among the whole population (~5–10%) exhibit such high levels of c-Fos and NFATc2, as shown in the cell density plot (Fig. 6B). The majority of cells express both transcription factors at intermediate to high levels (Fig. 6B, circles) with a frequency of IL-2-producing cells of 70–80%/bin (Fig. 6A, orange).

After normalizing and pooling the data from three independent experiments (Fig. 6C, left panel), we fitted the parameters of both interaction models. Three different tests of goodness revealed that in all cases (modeling of single experiments as well as the merged data set), the cooperative model explains IL-2 expression slightly, but consistently, better than the model describing the independent action of each transcription factor (supplemental Table S3). In particular, only the cooperative model can explain the low frequency of IL-2-producing cells (0–40%) at low to intermediate expression levels of c-Fos and NFATc2 per cell (Fig. 6C). Therefore, our conclusion is that the transcription factors c-Fos and NFATc2 act in a cooperative rather than in an independent manner to induce IL-2 expression.

**Manipulation of c-Fos Expression Confirms Causal Relationship between Amount of c-Fos and Probability of IL-2 Production per Cell**—To confirm that the transcription factor c-Fos is limiting for IL-2 production, we manipulated the physiological c-Fos expression levels using U0126, a specific MEK1/2 small molecular inhibitor. U0126 inhibits de novo synthesis of c-Fos after T cell stimulation. Inhibitor-treated and PMA/ionomycin-stimulated Th cells of six donors were stained and analyzed for the different transcription factors and IL-2 production.

The expression levels of c-Fos 5 h after strong stimulation decreases with U0126 in a dose-dependent manner (Fig. 7A, upper row), whereas the other transcription factors remained unaffected (NFATc2 and c-Jun) or even increased at very high concentrations (NF-κBp65) (supplemental Fig. S5). The c-Fos expression level does not shift to the level of unstimulated cells even when the highest concentration of U0126 is used. Remarkably, the expression level of c-Fos drops gradually rather than in an all-or-none manner.

Simultaneously, the frequency of IL-2 producers declines (Fig. 7A, lower row). However, the frequency of IL-2 producers already drops to zero at intermediate c-Fos expression levels. The sharp increase in the frequency of IL-2-producing cells with increasing c-Fos expression levels (Fig. 7A, right panel) suggests that a threshold amount of c-Fos defines whether a particular cell is able to express IL-2 or not. This finding supports the assumption that under strong stimulation conditions the heterogeneity of endogenous c-Fos expression levels within a memory Th cell population primarily determines whether cells produce IL-2 or not (Fig. 5B).

Heat maps of three-parameter stainings confirm that the c-Fos expression level decreases with increasing U0126 con-
centrations, whereas the NFATc2 expression level remains unaffected (Fig. 7B, lower row). Only cells with a c-Fos level above a certain threshold are able to produce IL-2. At 100 μM U0126, these cells represent just 2–3% of the cells within the memory Th population (Fig. 7B, upper row).

Our conclusion is that under strong T cell stimulation conditions, the endogenous c-Fos expression level ensures the robustness and variability of IL-2 expression within a memory Th cell population and mainly determines whether a single memory Th cell produces IL-2 or not. Our study demonstrates
DISCUSSION

Although the regulation of IL-2 expression in Th cells has been studied extensively for years (reviewed in Ref. 30), the quantitative behavior, the cell-to-cell variability, and the robustness of IL-2 decision making in primary Th cells remain unknown. The present study examined the impact of quantitative differences in the expression of the four main transcription factors NFATc2, NF-κBp65, c-Fos, and c-Jun (c-Fos/c-Jun = AP-1) on IL-2 expression. We investigated the consequences of physiological variation in transcription factor expression for IL-2 production within the subpopulation of ex vivo isolated human memory Th cells without manipulation, such as overexpression or silencing of the cells. Parallel measurement of the transcription factors and IL-2 at single-cell level enabled us, first, to discriminate between graded and all-or-none processes and, second, to correlate transcription factor levels with IL-2 levels and IL-2 decision making per cell.

Here we studied IL-2 expression under the special conditions that all memory Th cells are strongly stimulated either by PMA/ionomycin or anti-CD3/CD28 antibodies at high concentrations to obtain the maximum IL-2 production capability within the memory Th cell population. Depending on the individual donor, 40–70% of the memory Th cells have the potential to produce IL-2. We verified that under strong stimulation conditions, all four main transcription factors are activated and translocated into the nucleus in all memory Th cells. Under these conditions, we have provided several lines of evidence that c-Fos levels, and to a lesser extent NFATc2 levels per cell are limiting factors for IL-2 decision making in primary human memory Th cells. First, c-Fos and NFATc2 are expressed to a greater extent in IL-2-producing cells in comparison with IL-2-nonproducing Th cells. Second, the partitioning of cell numbers as a function of the levels of transcription factor revealed a positive correlation of c-Fos and NFATc2 with the number of IL-2-producing cells. Third, the endogenous level of c-Fos per cell is limiting for IL-2 production in ~40–50% of memory Th cells. Fourth, higher NFATc2 expression per cell enhances the number of IL-2-producing cells by 20–25% within the memory Th population. Fifth, mathematical modeling confirmed the cooperative effects of c-Fos and NFATc2 on IL-2 decision making. Finally, the MEK1/2 inhibitor U0126 inhibited the expression of c-Fos, and consequently the number of IL-2-producing cells, depending on concentration.

Our data pointing to a main role for c-Fos expression for IL-2 production are contradictory to the data from c-Fos knock-out mice. These mice revealed only a slightly decreased IL-2 expression compared with wild type mice (14). In the light of our data and data from c-Fos transgenic mice showing enhanced IL-2 production (17), we speculate that other Fos proteins might substitute for c-Fos in the c-Fos knock-out mice. Our single cell approach clearly showed the advantage of measurement of physiological transcription factor ranges, revealing that small differences of c-Fos levels between cells decide about the probability of IL-2 expression.

Our results that high NFATc2 levels increase the number of IL-2-producing cells confirmed that there has to be a certain threshold level of activated NFATc2 for IL-2 production in Th cells (31, 32). Dienz et al. (33) could show that a certain amount of active NFAT is required for IL-2 production in memory but not in naive Th cells. They used transgenic mice expressing a truncated, dominant-negative mutant of NFAT competing with NFATc2 and NFATc1 for binding to the Ser/Thr phosphatase calcineurin.

Despite the fact that NF-κBp65 and c-Jun are indispensable for IL-2 production (34–36), their natural variability does not affect the probability of IL-2 expression within the memory Th cell population. Therefore, these two transcription factors are noncritical components for IL-2 expression according to the definition of Feinerman et al. (20), because their physiological variance in expression does not measurably affect the response. However, it is important to note that they might be critical in their physiological concentrations in other cells as already shown for Treg cells (data not shown) or for the expression of other cytokines or activation-induced receptors.

Our approach using single cell data concerning transcription factor expression in primary human cells combined with partitioning of the amounts and subsequent correlation with readout parameters at the single cell level enabled us to investigate how quantitative variability per cell is translated into qualitative differences in cell responsiveness. However, the approach is limited by the availability of appropriate transcription factor antibodies that are specific and applicable for intracellular staining. Whether flow cytometry and Western blot data are comparable has to be checked for each individual antibody. Furthermore, the identification of one critical transcription factor does not preclude the identification of further regulatory transcription factors. These concerns have to be considered in studying the interesting question for the factor(s) that limit IL-2 production in c-Fos high memory Th cells. This fraction is ~10–20%. Potential transcriptional regulators depicted from recent publications are STAT-inducible repressors (37), NF-κBp50 (38), T-bet (39), Smad2/3 (40), and Runx1 (41).

Our results indicate that using ERK inhibitors to treat rheumatoid arthritis as proposed by Ohori (42) could be quite problematic. As we have shown, IL-2 production by Th cells is already blocked at an intermediate level of inhibition of c-Fos expression by an ERK inhibitor. In view of the fact that Th cells are the main source of IL-2, these conditions would limit the frequency of Foxp3+ Treg cells, because IL-2 is crucial for their induction and expansion. Recent publications showed that even the application of low dose IL-2 expands human Treg cell populations in patients with graft versus host disease (43) and HCV-induced vasculitis (44). Therefore, it has to be proven whether specific inhibitors for c-Jun or NF-κBp65 diminish the production of pro-inflammatory cytokines such as IFNγ or TNFα but leave the production of IL-2 intact. More generally, understanding the mechanisms that restrict variation in cell fate decision systems might enable new therapeutic intervention strategies and/or rule out others. This underlines the importance of revealing quantitative variations in physiological expression levels of transcription factors and their effects on...
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response parameters rather than studying qualitative changes using knock-out or transgenic mice, only.

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