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Differential Localization of T-bet and Eomes in CD8 T Cell Memory Populations

Laura M. McLane,* Pinaki P. Banerjee,† Gabriela L. Cosma,* George Makedonas,† E. John Wherry,* Jordan S. Orange,† and Michael R. Betts*

In mice, two T-box transcription factors, T-box expressed in T cells (T-bet) and eomesodermin (Eomes), drive the differentiation of CD8 T cell lineages; however, little is known regarding their role in human CD8 T cell differentiation. In this study, we characterized T-bet and Eomes expression and localization within human CD8 memory T cell populations. We find that T-bet and Eomes are broadly expressed in human memory CD8 T cells, with increasing levels of T-bet and Eomes strongly correlating with differentiation from central memory to effector memory and effector subpopulations. In resting T cells, T-bet levels directly correlate to subcellular localization, with a higher propensity for nuclear expression of T-bet within T-bet+ cells and predominantly cytoplasmic expression in T-bet− cells. In addition, Eomes is also localized to either the nucleus or the cytoplasm. Upon TCR stimulation, the percentage of T cells that express T-bet dramatically increases, whereas the percentage of cells expressing Eomes remains largely unchanged across all memory populations. Of interest, T-bet, but not Eomes, relocalizes to the nucleus in the majority of cells across all populations within 24 h post stimulation. These data indicate that T-bet and Eomes are likely regulated at the level of subcellular localization across all memory populations. These data suggest that the levels of T-bet and Eomes are important factors in determining the function and fate of a given T cell.

The online version of this article contains supplemental material.

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The online version of this article contains supplemental material.

Abbreviations used in this article: Eomes, eomesodermin; HDAC1, histone deacetylase 1; Hsp90, heat shock protein 90; MFI, median fluorescence intensity; RT, room temperature; T-bet, T-box expressed in T cells.

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Materials and Methods

Human cells

Donor PBMCs were collected after written, informed consent from the University of Pennsylvania’s Center for AIDS Research Human Immunology Core (Institutional Review Board 705906) in compliance with Institutional Review Board guidelines. PBMCs were cryopreserved in FBS (Hyclone, Logan, UT) containing 10% DMSO (Fisher Scientific, Pittsburgh, PA) and stored at −140°C until further use.

Flow cytometry analysis

Abs used in our flow cytometry studies are listed in Table I. Cryopreserved PBMCs were thawed and rested overnight at 37˚C, 5% CO₂, in complete medium: RPMI 1640 (Mediatech, Manassas, VA) supplemented with 10% FBS, 1% l-glutamine (Mediatech), and 1% penicillin–streptomycin (Lonza, Walkersville, MD), sterile filtered, at a concentration of 2 × 10⁶ cells per milliliter. Cells were washed with 1 × PBS, and α-CCR7 was added to each sample and incubated at 37°C, 5% CO₂, for 15 min. Samples were then stained with aqua amine-reactive viability dye for 10 min at room temperature (RT) in the dark. Surface stain Abs against various cell markers were next added to the cells and incubated for an additional 20 min at RT in the dark. Cells were washed with PBS containing 1% BSA (Fisher Scientific) and 0.1% sodium azide (Fisher Scientific). Cells were permeabilized for 20 min at RT using the Cytofix/Cytoperm Kit (BD Biosciences, San Jose, CA) and then washed twice with Perm/Wash Buffer (BD Biosciences). Intracellular cytokine staining Abs were then added to the cells and incubated for 1 h at RT in the dark. Cells were washed again with Perm/Wash Buffer and fixed in PBS containing 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). For each sample, 1,000,000 total events were acquired on a modified flow cytometer (LSRII; BD Immunocytometry Systems, Ashland, OR). Statistical analyses were done using GraphPad Prism software (Version 5.0a). When applicable, nonparametric Wilcoxon matched-paired test was performed.

Individual compensation tubes for each fluorophore in the experiment, were acquired on a modified flow cytometer (LSRII; BD Immunocytometry Systems, Ashland, OR). Statistical analyses were done using GraphPad Prism software (Version 5.0a). When applicable, nonparametric Wilcoxon matched-paired test was performed.

Cell fractionation and immunoblot analysis

Purified CD8 T cells were fractionated into nuclear and cytoplasmic compartments, using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL). Lysate concentrations for the nuclear and cytoplasmic compartments were determined using a BCA (bicinchoninic acid) Protein Assay Reagent Kit (Thermo Scientific). Then 20 μg total lysate from each compartment was analyzed for the presence of T-bet or Eomes. Standard methods for immunoblot analysis were used (19). T-bet was detected with a 1:200 dilution of a monoclonal α–T-bet Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Eomes was detected with a 1:500 dilution of a polyclonal α-Eomes Ab (Abcam, Cambridge, MA). As controls for efficient fractionation, a polyclonal α-histone deacetylase 1 (HDAC1) Ab (1:1000; Abcam) was used to detect the nuclear fraction, and a polyclonal α-heat shock protein 90 (Hsp90) Ab (Thermo Scientific) was used to detect the cytoplasmic fraction.

Confocal microscopy

CD8 T cells were prepared for fixed cell immunofluorescent confocal microscopy, as previously described (18). Abs used in our confocal microscopy studies are listed in Table I. Human α–T-bet, α–Eomes, and α–lamin A were used in the range of 0.5–2 μg/ml. A secondary goat α-rabbit IgG (H+L) conjugated to Alexa Fluor 568 (Molecular Probes, Grand Island, NY) was used to detect lamin A. Slides were then covered with 0.15-mm coverslips (VWR Scientific, Philadelphia, PA), using mounting media (Vectashield, Burlingame, CA) containing DAPI (Invitrogen, Carlsbad, CA) to visualize nuclear chromatin. Two-dimensional micrographs were obtained using a multilaser-based spinning disk confocal microscope (Zeiss).

Table I. Abs used in this study

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Activation and analysis of CD8 T cell memory populations

To study the effect of TCR activation on CD8 T cell populations, CD8 T cells from five donors were sorted, using a FACSAria (BD Immunocytometry Systems, San Jose, CA), into naive (CCR7+CD45RO+), central memory (CCR7+CD45RO−), effector memory (CCR7−CD45RO+), and effector (CCR7−CD45RO−) cells to obtain purified memory populations. Cell populations were rested overnight at 37°C, 5% CO₂, in complete medium: RPMI 1640 (Mediatech) supplemented with 10% FBS, 1% l-glutamine (Mediatech), and 1% penicillin–streptomycin (Lonza), sterile filtered. Cells were then cultured in 5 μg/ml CD3, 3 μg/ml CD28/CD49d, and 1 μg/ml BSA. Cells were collected at 0, 24, and 72 h post stimulation and stained for T-bet and Eomes, then analyzed on the ImageStream X. To determine if changes in blasting cells were different from changes in nonblasting cells, we performed statistical analysis on the area under each curve, followed by a paired Student t test. Paired t tests were also performed on localization analysis (Figs. 7C, 8C).
Results

T-bet and Eomes expression in CD8 T cell populations

Extensive studies in mice have suggested that T-bet and Eomes play key roles in effector function and long-term memory formation, respectively (1–3, 20, 21); however, the roles of T-bet and Eomes in human CD8 T cells remain unclear. As shown in Fig. 1A, human CD8 T cells display two T-bet+ populations, T-bethi and T-beto, whereas Eomes is bimodally distributed. Although the percentage of T-bet+ or Eomes+ cells varies greatly among individuals, these distinct populations for each transcription factor were observed in all donors we analyzed in our studies (data not shown).

To further characterize T-bet and Eomes expression, we assessed the expression of these transcription factors within naive (CCR7+CD45RO+), central memory (CCR7+CD45RO−), effector memory (CCR7−CD45RO+), and effector (CCR7−CD45RO−) CD8 T cells from normal donors (Table I). T-bet is undetectable in the majority of naive CD8 T cells (Fig. 1B). Approximately 25% of central memory express T-bet, and these cells are mainly T-bethi (Fig. 1B, 1C). A significant increase (p < 0.001) can be noted in the number of T-bet+ effector memory and effector cells, compared with central memory cells; however, a higher proportion of T-bethi cells can be found in the effector population. Similar to T-bet, Eomes is also undetectable in the majority of naive CD8 T cells (Fig. 1D). Although a small percentage of central memory cells are Eomes+, there is a significant increase (p < 0.001) in the proportion of Eomes+ effector memory and effector T cells, compared with central memory. Of interest, there is a statistically significant decrease (p < 0.001) in the proportion of Eomes+ effector cells compared with Eomes+ effector memory. In addition, Eomes was most highly expressed within effector memory cells compared with all other memory subsets (p < 0.001) (Fig. 1E). Taken together, these data reveal that effector CD8 T cells have the highest expression levels of T-bet, whereas Eomes is expressed in the highest frequency and expression levels in effector memory cells.

Localization of T-bet and Eomes in total resting human CD8 T cells

Whereas much characterization of T-bet and Eomes has focused on the presence or absence of these factors in various cell types, little work has investigated the localization, and thus the potential activity, of these factors. Because the function of many transcription factors is regulated by subcellular localization (9), we directly assessed the localization of T-bet and Eomes in human CD8 T cells. Using a high-resolution spinning disk confocal microscope, we visualized T-bet and Eomes in cells costained with DAPI and lamin A, which localizes to the inner nuclear envelope. We found that T-bet can be localized almost exclusively in the nucleus, as indicated by DAPI stain (Fig. 2A, top row); however, we also observed cells in which T-bet is localized throughout the cell (nuclear and cytoplasmic) and, in some cases, is almost exclusively localized to the cytoplasm (Fig. 2A, middle and bottom rows, respectively). Similarly, CD8 T cells also contain exclusively nuclear Eomes (Fig. 2B, top row); however, Eomes was also observed occasionally in both the nucleus and the cytoplasm alone (middle and bottom rows, respectively).

To confirm that T-bet and Eomes can be present in both the nucleus and the cytoplasm, CD8 T cells were fractionated into nuclear and cytoplasmic compartments and analyzed for the presence of T-bet or Eomes. Both T-bet and Eomes were detectable in the nuclear and cytoplasmic fractions of CD8 T cells (Fig. 3). As controls for efficient fractionation, the exclusively nuclear protein histone H4 was detected only in the nuclear fraction, whereas Hsp90, a cytoplasmic protein, was observed only in the cytoplasmic fraction. Taken together, these data show that T-bet and Eomes can be found in both the nuclear and cytoplasmic compartments within CD8 T cells and suggest that their functions might be regulated at the level of nuclear localization.

Higher levels of nuclear T-bet and Eomes correlate with CD8 T cell progression from memory to effector phenotypes

To better understand how the localization of T-bet and Eomes might contribute to CD8 memory subsets, we took advantage of Annis ImageStream technology, which allows for simultaneous population-based flow cytometry and fluorescence microscopy. Purified, resting CD8 T cells from five donors were stained for memory markers, CCR7 and CD45RO, as well as T-bet and Eomes. Cells were gated through memory markers, and T-bet and Eomes localization was investigated (Supplemental Fig. 1). Representative effector memory...
cells are shown in Fig. 4. T-bet<sup>hi</sup> cells contain either exclusively nuclear or nuclear and cytoplasmic T-bet (Fig. 4A), whereas T-bet<sup>lo</sup> cells contain exclusively nuclear, nuclear and cytoplasmic, or exclusively cytoplasmic T-bet (Fig. 4B). As with the confocal microscopy data, Eomes is detectable in the nucleus, nucleus and cytoplasm, or cytoplasm in CD8 T cells.

To begin investigating how T-bet and Eomes localization relates to different memory T cell phenotype, we next quantified T-bet and Eomes localization within naive, central memory, effector memory, and effector CD8 cells. To this end, T-bet<sup>+</sup> cells were separated into T-bet<sup>hi</sup> and T-bet<sup>lo</sup> populations (Supplemental Fig. 1). Using a masking function within IDEAS software (Amnis), the intensity of T-bet within the nucleus was plotted against the intensity of T-bet within the cytoplasm, allowing the quantification of differentially localized T-bet within memory populations. T-bet was not detectable in naive cells (data not shown). The percentage of T-bet<sup>+</sup> cells, and specifically the percentage of T-bet<sup>hi</sup> cells, increases as cells progress toward an effector phenotype (Fig. 5, top row; Supplemental Table I). In all CD8 T cell populations, T-bet<sup>hi</sup> cells contain T-bet that is predominantly exclusively nuclear, with a smaller fraction containing nuclear and cytoplasmic and exclusively cytoplasmic T-bet (Fig. 5, middle row; naive—data not shown). T-bet<sup>lo</sup> cells contain mixed populations of nuclear, nuclear and cytoplasmic, or cytoplasmic T-bet across all memory populations (Fig. 5, bottom row). The majority of T-bet<sup>+</sup> central memory cells are T-bet<sup>lo</sup>, and, of interest, ∼75% of these cells contain cytoplasmic T-bet, suggesting T-bet is not active in the majority of central memory cells. Approximately 70% of effector memory cells are T-bet<sup>+</sup>, with about a third of these being T-bet<sup>hi</sup> and and two thirds being T-bet<sup>lo</sup>. The majority of T-bet<sup>hi</sup> effector memory cells are nuclear, whereas the majority of T-bet<sup>lo</sup> cells are cytoplasmic. In contrast, ∼50% of effector cells are T-bet<sup>hi</sup>, and of these ∼85% contain exclusively nuclear T-bet, whereas ∼30% of T-bet<sup>lo</sup> cells contain some nuclear T-bet, suggesting T-bet is likely active in most effector cells.

In correlation with the data in Fig. 1D, Eomes is expressed in ∼15% of central memory, ∼50% in effector memory, and in just less than half of effector cells (Fig. 6A, top row; Supplemental Table I). Across all memory populations, between 60 and 75% of Eomes<sup>+</sup> cells contain some nuclear Eomes (Fig. 6A, bottom row), with central memory cells having the lowest amount of nuclear Eomes. Like T-bet, as cells become more effector-like, nuclear Eomes, as well as the amount of Eomes in a given cell, increases (Fig. 6B).

**T-bet expression and localization are modulated upon TCR activation of CD8 T cells**

We next investigated how generalized TCR activation affects T-bet expression and localization dynamics. To this end, purified CD8 T cells from five donors were sorted into naive, central...
memory, effector memory, and effector subsets based on CCR7 and CD45RO expression. Purified memory populations were activated with CD3, CD28, and CD49d for up to 48 h, and cells were collected at 24-h intervals. Blasting and nonblasting cells, as defined by size, were analyzed on the ImageStream. Within 24 h, T-bet expression was robustly induced in >80% of blasting cells following TCR stimulation in all memory phenotypes (Fig. 7A, solid line). The median fluorescence intensity (MFI) of T-bet also increases in blasting cells in all populations (Fig. 7B, solid line). Although nonblasting cells also can increase T-bet expression, blasting cells show a statistically significant increase in the observed overall percentage of T-bet+ cells, compared with nonblasting cells (Fig. 7A, dashed line; naive p = 0.002, central memory p = 0.017, effector memory p = 0.003, effector p = 0.003). Similarly, T-bet MFI in blasting cells from naive (p = 0.016), central (p = 0.013), and effector memory (p = 0.030) cells is significantly higher than in nonblasting cells, for which the MFI did not change over the course of 48 h following TCR stimulation across all populations (Fig. 7B, dashed line).

In addition to changes in T-bet expression, a significant increase was noted in the percentage of naive (data not shown) and effector memory cells containing nuclear T-bet that correlated with a decrease in the percentage of cells containing cytoplasmic T-bet (Fig. 7C). In naive cells, ~80% of T-bet+ cells contain nuclear T-bet following 24 h of stimulation (data not shown). Of interest, no significant increase was seen in the percentage of central memory and effector cells containing nuclear T-bet (Fig. 7C). We also observed varying amounts of nuclear and cytoplasmic T-bet over 48 h in each population, likely owing to new T-bet translation within the cytoplasm. There was no obvious difference in T-bet localization between blasting and nonblasting cells (data not shown).

To understand the dynamics of T-bet within the nuclear compartment, we next measured the MFI of T-bet within the nucleus.
We found that the MFI of T-bet within the nucleus of blasting cells increases in naive, central memory, and effector CD8 T cells (Fig. 7D, solid line; naive—data not shown), suggesting that T-bet is being actively shuttled into the nucleus, though at different levels, depending on memory type. These changes are significantly greater in blasting naive (\( p = 0.006 \)), central memory (\( p = 0.011 \)), and effector (\( p = 0.022 \)) cells than in nonblasting cells, for which the MFI of nuclear T-bet does not change (Fig. 7D, dashed line), indicating that the net overall amount of T-bet within the nucleus of these cells is not changing.

Taken together, these data reveal that TCR stimulation induces a robust increase in T-bet expression in all blasting memory populations. In addition, these data provide evidence to suggest that TCR signaling elicits cell-type specific changes to T-bet expression and localization.

Eomes expression and localization are not modulated by TCR stimulation in purified CD8 T cell populations

We next examined Eomes expression and localization in purified CD8 memory populations following TCR stimulation. In contrast to T-bet, the percentage of Eomes+ CD8 T cells, as well as the MFI of Eomes, does not significantly increase in either blasting or nonblasting cells following TCR activation in naive and effector cells within 48 h (Fig. 8A, 8B). We also analyzed the localization of Eomes and the MFI of nuclear Eomes within CD8 T cell memory populations and found there were no significant changes in locali-
zation induced by TCR stimulation in purified CD8 T cells. Taken together, these data reveal that, in contrast to T-bet, neither a net overall increase in Eomes expression nor changes in Eomes localization take place following TCR stimulation in purified CD8 T cells.

Discussion

In recent years, many studies have contributed to defining the mechanisms underlying control of CD8 T cell effector function in mice (21–24); however, little is known about CD8 effector differentiation in humans. In this study, we characterized the expression and localization patterns of the T-box transcription factors T-bet and Eomes in human CD8 memory T cell populations to begin dissecting their functions within these T cell subsets. In agreement with murine data (3), T-bet was observed in a graded expression pattern in which high levels of T-bet are associated with effector and some effector memory T cells and low levels of T-bet correlate with central memory T cells and some effector memory T cells.

A key and novel finding from our studies is that T-bet and Eomes can be localized outside the nucleus in CD8 T cells. Whereas T-bet has been observed in rare instances in the cytoplasm in dividing
cells (17), to our knowledge, our data provide some of the first evidence that T-bet and Eomes can be differentially localized within resting CD8 T cells. In addition, we show that high levels of T-bet and Eomes are more associated with nuclear localization than are low levels of T-bet or Eomes. Nuclear T-bet and nuclear Eomes are associated with effector and effector memory cells, suggesting T-bet and Eomes are active in these populations, whereas cytoplasmic T-bet and Eomes are associated predominantly with central memory phenotypes, indicating these factors are more likely to be inactive in this subset.

The compartmentalization of T-bet and Eomes to the nucleus or cytoplasm in specific cellular contexts is of particular consequence to T cell function. Evidence suggests that T-bet and Runx3, another transcription factor important to CD8 T cell differentiation (25), cooperatively bind the same genomic region to upregulate IFN-γ expression and also repress IL-4 expression in CD4 Th1 T cells (26). It is possible that T-bet and Eomes could also cooperatively bind the same gene promoters in CD8 T cells under specific conditions, depending on their cellular localization. Of note, recent studies suggest that there is a concentration-dependent binding of T-bet to specific sites within the genome (27). Exhausted cells are T-bet, and this decrease in T-bet expression correlates with a loss in the ability of T-bet to repress the inhibitory receptor, PD-1 (27). In addition, this hypothesis could also explain the contradiction that T-bet, which has been shown to repress IL-2, is expressed in IL-2–producing cells (28). The localization of T-bet in IL-2– or PD-1–expressing cells remains to be determined; however, on the basis of the low expression level of T-bet in these cells, it is likely that T-bet localization is key to this regulatory network.

Our studies also revealed important differences in the dynamics of T-bet and Eomes expression and localization. First, we show that generalized TCR stimulation robustly induces T-bet expression to >80% in blasting CD8 T cells within 24 h of TCR activation, suggesting that CD8 T cells alone have the ability to quickly respond to TCR stimulation to modulate T-bet. Whether TCR activation directly signals for T-bet upregulation, or if it signals for upregulation of a secondary signal that then induces T-bet expression, remains to be determined. Furthermore, T-bet localization changes following TCR activation across all memory populations except central memory, resulting in an increase in cells expressing nuclear T-bet at 24 h. Judging by the localization in central memory cells, we think it likely that upon TCR stimulation, pre-existing cytoplasmic T-bet is initially shuttled into the nucleus (Fig. 7C, %Cyto T-bet), and new T-bet is subsequently produced and also shuttled into the nucleus (%Nuc/Cyto T-bet). In this regard, the lack of an overall increase in the percentage of central memory cells containing exclusively nuclear T-bet is counterbalanced by the increase in cells expressing both nuclear and cytoplasmic T-bet.

In other words, T-bet is being shuttled into the nucleus, as evidenced by the increase in the MFI of nuclear T-bet, but these cells contain both nuclear and cytoplasmic T-bet, as indicated by the increase in the percentage of these cells. If this is the case, the localization data in other memory groups likely have different mechanisms or dynamics for T-bet relocation and upregulation. In contrast to T-bet, no significant changes to Eomes expression or localization in purified CD8 T cell populations occur following TCR stimulation. These data suggest that TCR stimulation alone is not sufficient to affect Eomes in purified CD8 T cells.

Overall, our data provide a baseline for future studies of T-bet and Eomes within the context of CD8 T cells and suggest a model wherein T-bet and Eomes localization can be modulated by TCR activation in specific cellular contexts. Such a mechanism ultimately has direct impact on the transcriptome and, consequently, overall phenotype and function of a given cell. In addition, our localization studies represent a novel way to investigate factors involved in immune cell functions that will undoubtedly contribute to the way future studies are developed when addressing the roles of lineage-defining transcription factors within the context of CD8 T cells. Most importantly, these studies indicate the presence of previously unknown mechanisms involved in the regulation of T-bet and Eomes function. Further studies of the mechanisms that sequester T-bet and Eomes in the nucleus or cytoplasm, and the signal(s) that trigger transport of T-bet and Eomes into or out of the nucleus, will provide a better understanding of T cell development and function. Such findings will undoubtedly be beneficial for future antiviral therapies by potentially unlocking the mechanism or mechanisms that could be exploited to manipulate CD8 lineage commitment and function.

Disclosures

The authors have no financial conflicts of interest.

References


Table I. Expression and Localization of T-bet and Eomes within CD8 Memory Populations

|        | T-bet$^{hl}$ % ± S.D. | T-bet$^{lo}$ % ± S.D. | Eomes % ± S.D. |
|--------|-----------------------|-----------------------|----------------
| **TCM** |                       |                       |                |
| Total  | 2.0 ± 0.9             | 26.9 ± 6.6            | 15.9 ± 4.8     |
| Nuclear| 75.1 ± 13.5           | 26.2 ± 2.9            | 60.6 ± 7.8     |
| Nuclear & Cytoplasmic | 11.6 ± 4.6 | 0.5 ± 0.3            | 5.5 ± 1.1      |
| Cytoplasmic | 12.4 ± 9.0 | 73.3 ± 3.8            | 33.8 ± 7.2     |
| **TEM** |                       |                       |                |
| Total  | 19.7 ± 10.8           | 49.6 ± 6.1            | 49.1 ± 15.6    |
| Nuclear| 67.6 ± 9.0            | 23.6 ± 5.3            | 67.9 ± 8.2     |
| Nuclear & Cytoplasmic | 13.5 ± 3.9 | 1.3 ± 1.1            | 2.3 ± 0.8      |
| Cytoplasmic | 19.2 ± 7.7 | 75.3 ± 5.3            | 29.4 ± 5.3     |
| **Effector** |                       |                       |                |
| Total  | 28.2 ± 3.7            | 27.4 ± 3.1            | 41.4 ± 14.7    |
| Nuclear| 85.5 ± 9.8            | 31.4 ± 6.9            | 72.2 ± 1.9     |
| Nuclear & Cytoplasmic | 10.0 ± 5.9 | 2.3 ± 1.6            | 2.0 ± 0.7      |
| Cytoplasmic | 4.5 ± 1.1 | 66.3 ± 8.1            | 25.8 ± 4.9     |
Supplemental Figure 1. Gating scheme for Imagestream analysis. Using purified CD8+ T cells obtained from the University of Pennsylvania Center for AIDS Research Human Immunology Core, singlets were delineated using the aspect ratio function versus the cell area of the brightfield image. Next, the gradient RMS brightfield function identified cells that were in focus. CD3+ DAPI+ cells were further subclassified, based on CCR7 and CD45RO expression, into naïve, central memory, effector memory, and effector CD8 T-cells. Within these subsets, T-bethi, T-beto, and T-bet− cells as well as Eomes+ and Eomes− cells were analyzed. Using a masking function within the IDEAS software, the percentages of cells that contain nuclear, nuclear and cytoplasmic, and cytoplasmic T-bet and Eomes were analyzed.