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OX40- and CD27-Mediated Costimulation Synergizes with Anti–PD-L1 Blockade by Forcing Exhausted CD8+ T Cells To Exit Quiescence

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Exhaustion of chronically stimulated CD8+ T cells is a significant obstacle to immune control of chronic infections or tumors. Although coinhibitory checkpoint blockade with anti–programmed death ligand 1 (PD-L1) Ab can restore functions to exhausted T cell populations, recovery is often incomplete and dependent upon the pool size of a quiescent T-bethigh subset that expresses lower levels of PD-1. In a model in which unhelped, HY-specific CD8+ T cells gradually lose function following transfer to male bone marrow transplantation recipients, we have explored the effect of shifting the balance away from coinhibition and toward costimulation by combining anti–PD-L1 with agonistic Abs to the TNFR superfamily members, OX40 and CD27. Several weeks following T cell transfer, both agonistic Abs, but especially anti-CD27, demonstrated synergy with anti–PD-L1 by enhancing CD8+ T cell proliferation and effector cytokine generation. Anti-CD27 and anti–PD-L1 synergized by downregulating the expression of multiple quiescence-related genes concomitant with a reduced frequency of T-bethigh cells within the exhausted population. However, in the presence of persistent Ag, the CD8+ T cell response was not sustained and the overall size of the effector cytokine-producing pool eventually contracted to levels below that of controls. Thus, CD27-mediated costimulation can synergize with coinhibitory checkpoint blockade to switch off molecular programs for quiescence in exhausted T cell populations, but at the expense of losing precursor cells required to maintain a response. The Journal of Immunology, 2015, 194: 125–133.

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or weakly replicating viruses fail to activate TNFR family receptors, enforcing costimulation experimentally through application of ligand fusion proteins or agonist Abs has shown the potential to enhance both primary and recall immunity (6).

The extent to which additional costimulation mediated via TNFR family receptors is beneficial under conditions favoring exhaustive differentiation of T cells is less clear. In murine models of chronic lymphocytic choriomeningitis (LCMV) infection, physiological expression of OX40 by virus-specific CD8+ T cells improves viral control (16). In contrast, continuous signaling via CD27 is implicated in driving even more profound exhaustion of virus-specific effectors (17). Agonistic Ab-mediated costimulation via 4-1BB can be detrimental or beneficial in promoting control of chronic LCMV according to the precise treatment schedule (18).

Thus, where expression of costimulatory ligands is already elevated or plentiful, driving further costimulation may have limited value. However, exhaustive CD8+ T cell differentiation may also occur under conditions in which costimulatory ligand expression is low, for example within tumors (19) or at late time points following allelogeneic stem cell transplantation (20). In the absence of help, nonlicensed APCs may lack the repertoire of costimulatory ligands required for full generation of productive immunity; in this context, coinhibitory signals could superevolve earlier and accelerate failure of chronically stimulated CD8+ T cells.

In this study, we have tested the hypothesis that provision of additional costimulation via TNFR family receptors under noninflammatory conditions will aid restoration of functions to exhausted CD8+ T cells. We find that agonistic Abs to OX40 and especially to CD27 synergize with anti–PD ligand 1 (PD-L1) by enhancing proliferation and effector cytokine generation. CD27-mediated costimulation synergized with coinhibitory checkpoint blockade to switch off molecular programs for quiescence in exhausted T cell populations, but this occurred at the expense of losing precursor cells required to maintain the response.

Materials and Methods

Animals

Female C57BL/6 (B6) and B6.SJL (CD45.1) mice were purchased from the Frederick Cancer Research facility and bred in house. MataHarI (Mh) TCR-transgenic mice on a B6.PL-Thy1a/Cy background were obtained from J. Dyson, Imperial College London (21). OVA+/- mice were bred in house (22). All procedures were approved by local institutional research committees and conducted in accordance with National Institutes of Health or United Kingdom Home Office Animals (Scientific Procedures) Act of 1986.

PD-L1, OX40, OX40L, and CD27 Abs

Anti-mouse PD-L1 (10F9G2), anti-OX40 (OX86), anti-CD27 (AT124-1), and their respective isotype controls have been described previously (23–26).

Bone marrow transplantation and adoptive T cell transfer

Male B6 mice received 9 Gy irradiation, followed by receipt of 5 × 10^6 female B6 bone marrow cells. Seven days later, 1 × 10^7 Mh CD8+ transgenic T cells with or without additional 2.5 × 10^6 female B6 polyclonal CD4+ T cells were transferred to bone marrow transplantation (BMT) recipients (20). Individual CD4+ and CD8+ donor populations were selected using CD4/CD8 T cell isolation kits (Miltenyi Biotec). Uptake of BrdU was used to examine the turnover of cells in vivo at later time points by administration of 0.8 mg/ml BrdU (Sigma-Aldrich) in the water of recipient mice over 7 d prior to analysis.

Abs and flow cytometry

The following cell surface Abs (purchased from eBioscience, San Diego, CA) and their respective isotype controls were used: anti-CD8a (53-6.7), anti-CD27 (LG.7F9), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD107a (1D4B), anti-Thy1.1 (HIS51) anti–PD-1 (J43), and anti-OX40 (OX86).

Anti-Vß8.3 TCR (1B3.3) was purchased from BD Biosciences (Oxford, U.K.). Direct intracellular staining was carried out using anti-perforin (OMAK-D), anti–T-bet (4B10), anti–Eomes (Dan11mag), and isotype controls, purchased from eBioscience (San Diego, CA), or anti-granzyme B (GRB05) from Life Technologies. Following brief peptide restimulation, intracellular staining was performed using anti–IFN-γ (XMG1.2) or anti–TNF-α (MP6-XT22) together with the appropriate isotype controls (BD Biosciences, Oxford, U.K.). Intramural staining for BrdU was carried out using anti–BrdU-allophycocyanin flow kit (BD Biosciences), according to the manufacturer’s instructions. To detect CD107a, cells were restimulated for 4 h in the presence or absence of 1 µM UTY peptide with Golgi-Stop (BD Pharmingen) in the presence of anti-CD107a or isotype control. Cells were then resurface stained with anti-CD107a or isotype. Flow cytometric analysis was performed on a LSRFortessa or FACS Canto II (BD Biosciences), and cell counting was performed on a Coulter Counter (Beckman Coulter).

Intracellular cytokine staining

Cells from spleen or blood were analyzed for Ag-specific IFN-γ or TNF-α release by ex vivo intracellular staining. Briefly, cells were cultured in the absence or in the presence of 1 µM of the peptide UTY (WMHHINMDLI) or irrelevant peptide (OVA SIINFEKL) for 4 h (ProImmune or Peptide Protein Research), and brefeldin A (GolgiPlug; BD Pharmingen) was added either at the start or for the last 2 h. Cells were then surface stained for CD8 and Vß8.3, or relevant congenic markers, fixed, permeabilized, and then analyzed for intracellular cytokine staining by addition of the appropriate Ab.

Gene expression analysis

mRNA was isolated using the RNeasy Micro Kit, followed by cDNA generation using the RT2 PreAMP cDNA Synthesis Kit (both Qiagen). Gene expression analysis was performed using the RT2 Profiler T Cell Anergy and Immune Tolerance PCR Array by quantitative PCR using Actb, Gusb, and Hsp90ab1 housekeeping genes for normalization (Qiagen). Raw threshold data generated by PCR was uploaded to a dedicated web portal for further analysis (http://www.sabiosciences.com/prcarrarydataanalysis.php).

Statistical analyses

Statistical analyses were performed using the unpaired t test (two tailed). A p value <0.05 was considered to be significant (*p < 0.05, **p < 0.01, ***p < 0.001).

Results

Aagonistic costimulation via OX40 synergizes with anti–PD-L1 to recover functions of helpless, exhausted CD8+ T cells

We have shown previously that TCR-transgenic Mh CD8+ T cells (specific for the male Ag, UTy) become exhausted following delayed transfer to male, MHC-matched BMT recipients (20). Administration of anti–PD-L1 can partially rescue the functions of exhausted Mh CD8+ T cells under conditions in which they are cotransferred with polyclonal female CD4+ T cells (20). Because CD4+ T cells can protect against exhaustion in models of chronic viral infection (27), we first evaluated whether exhaustion of helpless Mh CD8+ T cells could similarly be reversed following anti–PD-L1. As shown in Fig. 1A, both CD4 helped and unhelped Mh CD8+ T cells (Thy1.1+ Vß8.3) demonstrated similar reductions in their capacity to produce IFN-γ over time following their transfer to male B6 BMT recipients. However, Mh CD8+ T cell expression of PD-1 was higher in the unhelped versus the helped experimental group (Fig. 1B), and, although anti–PD-L1 treatment could enhance proliferation from baseline in helpless Mh CD8+ T cells, it had little effect upon generation of IFN-γ (Fig. 1C). This was in contrast to helped conditions, in which, as we have previously reported (20), anti–PD-L1 treatment increased both proliferation and IFN-γ generation. Because the amount of male Ag remains constant throughout the experiment, these data show that CD4+ T cells can protect CD8+ T cells from exhaustion through effects that are independent of any change in overall Ag load.
In initial experiments to examine the effect of additional costimulation upon rescue of exhausted Mh CD8\(^+\) T cells, we first evaluated the expression of TNFR family members that are up-regulated in response to TCR activation (OX40, 4-1BB). Compared with naive input cells, we observed increased expression of OX40, which was equivalent upon both helped and unhelped CD8\(^+\) T cells at day 42 following transfer (Fig. 2A). To test the effect of enforcing OX40 costimulation in helped compared with unhelped Mh CD8\(^+\) T cells, we administered an agonist anti-OX40 mAb (OX86) on day 35 following T cell transfer, alone or in combination with anti–PD-L1 given on days 36 and 39, and evaluated the response at day 42. Under both helped and unhelped conditions, anti-OX40 alone given had no effect upon proliferation (as estimated by BrdU incorporation) or IFN-\(\gamma\) production by Mh CD8\(^+\) T cells (Fig. 2B, 2C, Supplemental Fig. 1A). However, when anti-OX40 was combined with anti–PD-L1 blockade, we observed synergistic increases in Mh CD8\(^+\) T cell proliferation that were evident in helpless, but not helped cells (Fig. 2B, 2C). In the helpless setting, the combination of anti–PD-L1 and anti-OX40 also synergized to increase the absolute numbers of IFN-\(\gamma\)\(^+\) Mh CD8\(^+\) T cells in the spleen, although these effects were relatively modest when evaluated on a per-cell basis (Fig. 2B, 2C, Supplemental Fig. 1A). The synergy for effector cytokine expression in the combined treatment group was limited to IFN-\(\gamma\); anti-OX40 alone had no effect upon TNF-\(\alpha\) generation, whereas anti–PD-L1 induced a minor increase from baseline with no further increase upon the addition of anti-OX40 (Supplemental Fig. 1B). No increases were observed in the frequency of Mh CD8\(^+\) T cells dual staining for IFN-\(\gamma\) and TNF-\(\alpha\) in any treatment group (Supplemental Fig. 1B). Because OX40 is expressed constitutively on murine T regulatory cells and is inducible on other cells, including NK and NKT cells (7), we also tested whether the effect of combined anti–PD-L1 and anti-OX40 would still occur under conditions in which Mh CD8\(^+\) T cells expressed OX40, but other immune cell populations did not. Thus, we transferred unhelped Mh CD8\(^+\) T cells to male BMT recipients, as set out in Fig. 2B, but reconstituted irradiated male recipients with female OX40\(^{-/-}\) bone marrow. Under these experimental conditions, anti–PD-L1 treatment alone had no effect, whereas anti-OX40 and anti–PD-L1 induced significant increases in absolute numbers of IFN-\(\gamma\)\(^+\) Mh CD8\(^+\) T cells, indicating an intrinsic effect of costimulation upon the transferred CD8\(^+\) T cell population (Supplemental Fig. 1C).

**Agonistic costimulation via CD27 is more effective than OX40 in the initial reversal of CD8\(^+\) T cell exhaustion**

CD27 is another TNFR family member that differs from OX40 in that its expression is constitutive upon naive CD8\(^+\) T cells. As shown in Fig. 3A, CD27 was expressed to a similar extent upon both input naive and unhelped exhausted Mh CD8\(^+\) T cells at day 42 (Fig. 3A). In a similar approach to the experiments outlined in Fig. 2, we applied agonistic anti-CD27 alone or in combination with anti–PD-L1 to male BMT recipients adoptively transferred with unhelped Mh CD8\(^+\) T cells 5 wk earlier. In contrast to anti-OX40, we found that anti-CD27 given alone significantly improved proliferation of unhelped Mh CD8\(^+\) T cells, although it had no effect upon IFN-\(\gamma\) generation (Fig. 3B, 3C, Supplemental Fig. 1). When anti-CD27 was combined with anti–PD-L1, additive effects upon proliferation and synergistic increases in IFN-\(\gamma\) expression were noted (Fig. 3B, 3C, Supplemental Fig. 1A). The synergy for effector cytokine expression in the combined treatment group was limited to IFN-\(\gamma\); both anti-CD27 and anti–PD-L1 alone induced minor increases in TNF-\(\alpha\) generation, with no further increases in the combined treatment group (Supplemental Fig. 1B). No increases were observed in the frequency of Mh CD8\(^+\) T cell dual staining for IFN-\(\gamma\) and TNF-\(\alpha\) in any treatment group (Supplemental Fig. 1B). Similar to our findings with anti-OX40 and anti–PD-L1, synergy between anti-CD27 and anti–PD-L1 was only observed under helpless conditions (Supplemental Fig. 1D). To compare the effects of combining anti–PD-L1 with each TNFR Ab, we performed direct comparisons of anti-OX40 or anti-CD27 in combination with anti–PD-L1. As shown in Fig. 4, anti-CD27 and anti-OX40 in combination with anti–PD-L1 were similarly effective in promoting a proliferative response in Mh CD8\(^+\) T cells and in enhancing effector function as evaluated by CD107a expression. However, anti–PD-L1 plus anti-CD27 were more effective than anti–PD-L1 and anti-OX40 in enhancing the effector function of Mh CD8\(^+\) T cells as evaluated by IFN-\(\gamma\) upon peptide stimulation (Fig. 4).
Molecular profiling of T cell responses to TNFR costimulation in the presence or absence of coinhibitory checkpoint blockade

To identify potential mechanisms underlying the synergy between OX40 and CD27 agonistic Abs and anti–PD-L1, we extracted mRNA from flow-sorted Mh CD8⁺ T cells derived from mice, 7 d following the commencement of individual or combined Ab combinations (day 42, n = 4 mice/group and then performed quantitative RT-PCR for 84 individual genes linked to T cell tolerance and effector functions. As shown in the heat map in Fig. 5A, the gene expression profiles of Mh CD8⁺ T cells derived from mice receiving anti-CD27 and anti–PD-L1 segregated from the other groups, although there was some overlap, primarily with the anti-CD27 group and the combined anti-OX40/anti–PD-L1 group. Within the subset of genes showing reduced transcription in the anti-CD27 and anti–PD-L1 group, we found that a significant number was involved in quiescence and/or anergy, including genes encoding transcriptional repressors (Foxp1, Foxp3, Egr2, Egr3, Ing4), E3 ubiquitin ligases (Ich, Rnf128), and other genes encoding proteins preventing TCR-proximal signaling (Dgka, Dgkz) or providing coinhibitory signals (Cta4, Pdcd1, Btla). Using a ≥2.0-fold reduction cutoff, we evaluated how these genes were affected in each of the experimental groups (Fig. 5B). All of the treatment groups were characterized by a core signature of reduced Egr3 expression and, with the exception of the anti–PD-L1 group, reduced Foxp3 expression. Egr2, an Egr3-related transcription factor gene required for the induction of several other anergic factors (28–30), was reduced in the anti-CD27 group and both the combined treatment groups. In addition, the combined anti-CD27 and anti–PD-L1 group showed reductions in the expression of a more extensive set of anergy-related genes (Fig. 5B). The number of downregulated genes was not as wide in the combined anti-OX40 and anti–PD-L1 group despite the demonstration of significant synergy in terms of proliferation; however, this group was additionally characterized by reduced expression of the coinhibitory receptor Btla (an effect that was also seen, but to a lesser extent in the anti-OX40 group; Fig. 5B and data not shown). To evaluate this gene expression pattern in more detail, we examined the expression of a panel of 10 genes that have been shown individually to be necessary for anergy or quiescence (28–30). When anti-CD27 was combined with anti–PD-L1, the entire panel of anergy and quiescence genes showed reduced expression with a clear synergistic pattern. In the combined anti-OX40 and anti–PD-L1 group, marked synergy was observed for Egr2 down-regulation with further additive effects upon Rnf128 repression.
In contrast, with the exception of Egr3, none of the anergy genes was downregulated in the PD-L1 group. We also extended this analysis to evaluate the entire gene set using a 2.0-fold change cutoff (Supplemental Fig. 2). Using these criteria, it was noteworthy that the changes in the anergy- and quiescence-related genes were not paralleled by reciprocal increases in the expression of genes encoding effector molecules or the transcriptional regulators that induce them when considered at a whole population level. Indeed, the expression of several regulator genes required for effector differentiation (Jak3, Gata3, Stat6, Tbx21, Icos, Irf4, Fos, Jun) was in fact reduced in Mh CD8+ T cells derived from mice receiving anti-CD27 and anti–PD-L1. Loss of T-bethighEomeslow cells by combined treatment with anti-CD27 and anti–PD-L1 leads to eventual contraction of the effector pool

In the model of CD8+ T cell exhaustion following chronic LCMV infection, a quiescent T-bethighEomeslowPD-1low precursor population is required for maintaining residual immunity to Ag and for restoration of functions in response to PD-1 checkpoint blockade (5, 31). Because anti-CD27 alone, and in combination with anti–PD-L1, switched off a number of anergy- or quiescence-related genes, we wanted to determine how this would impact upon the precursor population and maintenance of immunity long-term. We therefore conducted experiments in which we tracked Mh CD8+ T cell function in relation to T-bet and Eomes expression at both early and late time points following Ab treatment. Using the same treatment schedule as Fig. 3, but starting Ab treatment at day 59 following unhelped T cell transfer, we again observed a synergistic increase in the frequency and absolute numbers of peripheral blood Mh CD8+ T cells in the combined anti-CD27 and anti–PD-L1 group (Fig. 6A, 6B). As in previous experiments, the combined Ab treatment led to a synergistic increase in the frequency of IFN-γ+ cells following exposure overnight to UTY peptide, with gates set according to irrelevant peptide. Bottom, Representative histograms showing the percentage of Mh CD8+ T cells incorporating BrdU. (C) Graphs show mean percentage ± SEM of Mh CD8+ T cells that had incorporated BrdU, mean percentage ± SEM of Mh CD8+ T cells that produced IFN-γ, and mean ± SEM absolute numbers of Mh CD8+IFN-γ+ cells/spleen. Data are pooled from two independent experiments. Statistical comparisons performed using two-tailed, unpaired Student t test: *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 3.** Effect of agonistic anti-CD27 and/or blocking anti–PD-L1 Ab upon unhelped Mh CD8+ T cell effector functions. (A) From experiments of similar design to those in Fig. 2, representative histograms show CD27 expression upon naive input Mh CD8+ T cells or on day 42 (open histograms) following transfer to irradiated male mice and isolation from recipient spleens. Filled histograms show isotype control staining. Numbers indicate mean fluorescence intensity. (B) BMT recipients were given anti-CD27 on day 35 following unhelped Mh CD8+ T cell transfer (n = 8) or 200 μg anti–PD-L1 blocking Ab on days 36 and 39 (n = 9) or both Abs (n = 9). Control mice received the same number of i.p. injections with the relevant isotype control (n = 7). Top, Representative contour plots show IFN-γ production by Mh CD8+ T cells following exposure overnight to UTY peptide, with gates set according to irrelevant peptide. Bottom, Representative histograms showing the percentage of Mh CD8+ T cells incorporating BrdU. (C) Graphs show mean percentage ± SEM of Mh CD8+ T cells that had incorporated BrdU, mean percentage ± SEM of Mh CD8+ T cells that produced IFN-γ, and mean ± SEM absolute numbers of Mh CD8+IFN-γ+ cells/spleen. Data are pooled from two independent experiments. Statistical comparisons performed using two-tailed, unpaired Student t test: *p < 0.05, **p < 0.01, ***p < 0.001.

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Discussion

In this study, we have shown that anti-OX40– and anti-CD27–mediated costimulation synergized with coinhibitory checkpoint blockade to restore functions of exhausted, helpless CD8+ T cells. When combined with anti–PD-L1, stimulation of T cells through OX40 and especially CD27 primarily acted to block molecular programs for anergy or quiescence, driving CD8+ T cells to undergo rapid proliferation and terminal differentiation. However, transient improvements in effector functions occurred at the expense of loss of precursor cell populations capable of sustaining the response.

As in models of chronic viral infection, unhelped HY-specific CD8+ T cells become more exhausted than helped cells and less amenable to rescue through anti–PD-L1 blockade alone. These findings are also consistent with the concept that CD4+ T cells are less prone to exhaustive differentiation than CD8+ T cells (32) and thus capable of providing helper signals at the priming and/or maintenance phase of the response (27). Because the amount of male Ag is fixed during the development of HY-specific CD8+ T cell exhaustion following BMT, the capacity of CD4+ T cells to mitigate against CD8+ T cell exhaustion is independent of any effect upon the overall Ag load. Lack of help was linked to higher expression of PD-1 upon the exhausted Mh CD8+ T cell repertoire, and this is likely to explain the relative failure of helpless cells to respond to anti–PD-L1. The effect of CD4+ T cells upon PD-1 expression could be direct, for example through the synthesis of IL-2, which can limit upregulation of PD-1 upon CD8+ memory T cells (33), or indirect, through licensing of APCs (34). Lack of CD4+ T cell help is also a major obstacle to successful immunotherapy of cancer (35). In a tumor model characterized by ineffective Ag presentation via MHC class II (36), we have also observed synergy between anti-OX40 and anti-CD27 with anti–PD-L1. Thus, following vaccination with irradiated B16 melanoma cells expressing Flt3-ligand, addition of either anti-OX40 or anti-CD27 to anti–PD-L1 synergized to enhance tumor infiltration by endogenous T cells and significantly delay tumor growth (S. Buchan and A. Al-Shamkhani, unpublished data). The finding that tumor-reactive CD8+ T cells within human melanomas frequently coexpress PD-1 and TNFR family costimulatory molecules (37) suggests that this approach may have clinical application.

OX40- or CD27-mediated costimulation activates several pathways downstream of the TCR that are inhibited via PD-1 signaling, including PI3K-Akt, NF-κB, and NFAT (38). In addition numbers of IFN-γ-γ+ Mh CD8+ T cells/spleen in the single anti–PD-L1 Ab-treated mice were greater than controls, indicating a long-term protective effect of this Ab against exhaustion. To determine how spleen Mh CD8+ T cell function at day 120 correlated with expression of T-bet and Eomes, we performed intracellular staining for each transcription factor. Consistent with the hypothesis that anti-CD27 was driving further loss of precursors required for maintaining Ag responsiveness, the surviving Mh CD8+ T cells in both the combined anti-CD27 and anti–PD-L1 group and the single anti-CD27 group showed significant skewing to a terminally differentiated T-bethighEomeshigh phenotype with a relative loss of cells that were T-bethighEomeslow (Fig. 6E). Furthermore, in a pooled analysis of all the groups, we observed a significant negative correlation between the percentage of cells that were Eomeshigh and the percentage of cells capable of generating IFN-γ (Fig. 6F). Thus, additional CD27 costimulation enables temporary rescue of exhausted CD8+ T cells, but at the expense of driving their terminal differentiation and eventual loss of function.
tion, PD-1 ligation also interrupts proximal TCR signaling through mechanisms that require recruitment of Src homology region 2 domain–containing phosphatase 2 to an immunoreceptor tyrosine-based switch motif contained within its cytoplasmic domain (39). For the most part, we observed that the interaction between OX40- and especially CD27-induced costimulation was synergistic with anti–PD-L1, indicating that TNFR family–mediated signaling was acting on molecular pathways distinct from those inhibited by PD-1. The lack of synergy in the helped setting would also imply that signaling via OX40 or CD27 is somehow suboptimal in unhelped CD8+ T cells, although whether this is a T cell–intrinsic effect and/or the result of reduced ligand expression requires further study. Following cotreatment with anti-CD27 and anti–PD-L1 especially, several genes encoding proteins promoting anergy or quiescence were sharply downregulated. This finding was unexpected because the transcriptional programs that underlie exhaustion and anergy are often viewed as being distinct (40), with lack of quiescence being a key property assigned to exhausted T cells when the population is examined as a whole (4). Indeed, the behavior of the Mh CD8+ T cells upon adoptive transfer to male recipients did not parallel in vivo models of anergy in which T cell functions are impaired almost immediately upon Ag encounter (41). Instead, more akin to models of exhaustion, the functions of Mh CD8+ T cells were progressively lost over time (Supplemental Fig. 4). Nevertheless, as the proinflammatory effects of irradiation diminish, it is possible that reduced levels of costimulation (signal 2) also induce overlapping anergic molecular programs that contribute to the observed T cell dysfunction.

When evaluated at a whole population level, we did not observe reciprocal changes within the anergy and effector gene sets in

FIGURE 5. Effect of anti-OX40 and anti-CD27 alone or in combination with anti–PD-L1 upon expression of genes linked to tolerance or effector function. Experimental design as set out in Figs. 2–4. On day 42, unhelped Mh CD8+ T cells were flow sorted to high purity from recipient spleens and mRNA extracted (n = 3 mice in controls; n = 4 mice/group in Ab treatment groups). (A) Heat map showing quantitative RT-PCR for 84 genes tested (Materials and Methods). (B) Venn diagram showing pattern for >2.0-fold reduction in anergy/quiescence genes according to treatment group. (C) Graphs showing fold change expression compared with controls for a panel of 10 anergy quiescence-related genes. The p values were calculated based on an unpaired t test of the replicate 2^(-Ct) values for each gene in the control group and treatment groups: *p < 0.05, **p < 0.01, ***p < 0.001.
T cells following combined coinhibitory blockade and enforced CD27-mediated costimulation. Reduced expression of anergy-related factors would be predicted to enhance TCR-proximal signal transduction leading to downstream activation of Ras, ERK, and JNK pathways that in turn induce effector differentiation (29). However, several downstream master regulators of effector gene expression were in fact moderately downregulated in the combined treatment group when evaluated directly ex vivo despite the transiently increased capacity of T cells to undergo degranulation or generation of IFN-γ upon restimulation. Without performing single-cell analyses of gene expression, we cannot exclude the possibility that the lack of reciprocity reflects the fact that the population evaluated is mixed, including cells that remain exhausted as well as those with restored functions. Alternatively, the quantitative PCR may miss the effects of posttranscriptional regulation of the relevant genes. However, recent studies have also revealed different patterns of connectivity in exhausted compared with effector or memory cells, such that specific transcription factors (e.g., T-bet) can possess highly variable transcriptional interactions according to their cellular context (4). Thus, removal of anergy factors and enhanced TCR-coupled signal transduction may be insufficient to fully restore effector programs that have become disconnected in the setting of continual TCR stimulation.

Although combined anti-CD27 and anti–PD-L1 led to robust increases in proliferation and measurable gains in effector function upon restimulation, these effects were not sustained. It is known that tolerant cells induced to undergo proliferation in response to lymphopenia also transiently improve their effector functions but then switch back to their tolerant state as epigenetic changes required for tolerance are re-established (42); it is possible that a similar mechanism applies in the setting of exhaustion. However, an alternative and nonexclusive mechanism suggested in this study is that the loss of quiescence factors induced by anti-CD27 with or without anti–PD-L1 eventually leads to a loss of precursor cells and consequent reductions in the total pool size of Ag-specific T cells as effectors reach their Hayflick limit and undergo replicative senescence (43). The fact that the effector pool was also eventually diminished in the group treated with anti-CD27 Ab alone suggests that reductions in only a subset of anergy factors are sufficient for the loss of T-bet high precursors to

**FIGURE 6.** Effect of agonistic anti-CD27 with or without anti–PD-L1 Ab upon unhelped Mh CD8+ T cell T-box factor expression and long-term effector functions. Experimental design as set out in Fig. 3, except that anti-CD27 was given on day 59 and anti–PD-L1 on days 59 and 62, with isotype control Abs given on the same days (n = 4/group). (A) Frequency of blood Mh CD8+ T cells (as percentage of live gate) following indicated Ab treatment. Statistical comparisons are for combined treatment group versus isotype control. (B and C) Analyses performed on day 65 in peripheral blood. (B) Left, Mean ± SEM absolute number of blood Mh CD8+ T cells. Middle, Mean percentage ± SEM of blood Mh CD8+ T cells that produced IFN-γ. Right, Mean ± SEM absolute number of Mh CD8+IFN-γ+ cells/ml blood. (C) Graphs showing mean percentage of blood Mh CD8+ T cells that were T-betlowEomeslow (left) or T-bethighEomeslow (right) on day 65 following transfer. (D–F) Analyses performed on day 120 in spleen. (D) Mean percentage ± SEM of Mh CD8+ T cells that produced IFN-γ (left) and mean ± SEM absolute numbers of Mh CD8+IFN-γ+ cells/spleen (right) at day 120 following transfer. (E) Graphs showing mean percentage of spleen Mh CD8+ T cells that were T-bethighEomeslow (left) or T-bethighEomeshigh (right) on day 120 following transfer. (F) Scatter plot showing correlation between frequency of IFN-γ+ Mh CD8+ T cells (x-axis) versus frequency of cells that were Eomes+ (y-axis). Data are representative of two independent experiments with similar design. Statistical comparisons performed using two-tailed, unpaired Student t test: *p < 0.05, **p < 0.01, ***p < 0.001.
occurs. Future studies addressing the potential of combined coinhibitory checkpoint blockade and costimulation therapies will therefore need to examine in detail how restoration of exhausted T cell functions affects the composition of the emerging repertoire according to markers of replicative potential such as T-box transcription factor expression and telomere length. The risk that such therapies deplete the effector pool still further may be greatest when levels of Ag and/or costimulation are already high.

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Disclosures

G.J.F. has patents and receives patent royalties on the PD-1 pathway. The remaining authors have no financial conflicts of interest.

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