Cutting Edge: A Double-Mutant Knockin of the CD28 YMNM and PYAP Motifs Reveals a Critical Role for the YMNM Motif in Regulation of T Cell Proliferation and Bcl-xL Expression

Jonathan S. Boomer, Christine M. Deppong, Dulari D. Shah, Traci L. Bricker and Jonathan M. Green

*J Immunol* 2014; 192:3465-3469; Prepublished online 17 March 2014; doi: 10.4049/jimmunol.1301240

http://www.jimmunol.org/content/192/8/3465

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2014/03/17/jimmunol.1301240.DCSupplemental

**References**

This article cites 33 articles, 18 of which you can access for free at:

http://www.jimmunol.org/content/192/8/3465.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Cutting Edge: A Double-Mutant Knockin of the CD28 YMNM and PYAP Motifs Reveals a Critical Role for the YMNM Motif in Regulation of T Cell Proliferation and Bcl-x\textsubscript{L} Expression

Jonathan S. Boomer,* 1 Christine M. Deppong,* 1 Dulari D. Shah,* Traci L. Bricker,* and Jonathan M. Green*†

CD28 is a critical regulator of T cell function, augmenting proliferation, cytokine secretion, and cell survival. Our previous work using knockin mice expressing point mutations in CD28 demonstrated that the distal proline motif was primarily responsible for much of CD28 function, whereas in marked contrast to prior studies, mutation of the PI3K-binding motif had little discernible effect. In this study, we examined the phenotype of mice in which both motifs are simultaneously mutated. We found that mutation of the PYAP motif unmasks a critical role for the proximal tyrosine motif in regulating T cell proliferation and expression of Bcl-x\textsubscript{L} but not cytokine secretion. In addition, we demonstrated that, although function is more severely impaired in the double mutant than in either single mutant, there remained residual CD28-dependent responses, definitively establishing that additional motifs can partially mediate CD28 function. The Journal of Immunology, 2014, 192: 3465–3469.

Costimulation by CD28 initiates a complex cascade of events that ultimately results in enhanced T cell activation and augmented effector cell function (1). The precise signaling pathways used by CD28 have been difficult to unravel, and whether CD28 acts exclusively as an amplifier of TCR-mediated signals or whether it initiates a unique pathway has remained controversial (2). Within the cytoplasmic tail two regions have been of particular interest: a membrane proximal YMNM motif and a distal PYAP motif. Both regions have been demonstrated to complex with several kinases and adaptor proteins, with some proteins being able to bind to either or both motifs through SH2 and/or SH3 domain interactions (3–8). The binding of PI3K to the YMNM motif and Src family kinases to the PYAP motif have been thought to be the major initiators of signaling. Each motif was shown to contribute to CD28-dependent effects, including the regulation of IL-2 production and cell survival, although there have been significant discrepancies depending upon the experimental system used.

To address the role and relative importance of each motif, we previously generated knockin mice that express mutations of either the proximal tyrosine (CD28-Y170F)–based motif or the distal proline (CD28-AYAA)–based motif (9, 10). Despite an abundance of literature suggesting an essential role for activation of PI3K by the Y170 motif (11–13), we were unable to detect a biologically meaningful phenotype in mice in which this motif had been mutated. In contrast, mutation of the distal proline motif resulted in a marked impairment of function, although costimulation with anti-CD28 Abs still resulted in an increase in proliferation, and an in vivo model of allergic airway inflammation was essentially normal.

Given the preservation of CD28-dependent responses, we hypothesized that compensatory signaling through the distal proline motif might account for the relatively preserved function of the CD28-Y170F cells and, similarly, that the remaining CD28-dependent responses in the CD28-AYAA mice might be due to signaling initiated by the intact Y170 motif. To formally test this, we generated knockin mice in which both the Y170 and PYAP motifs were mutated (CD28-Y170F/AYAA). We found that there was a reproducible decrease in proliferation, and an in vivo model of allergic airway inflammation was essentially normal.

Therefore, we conclude that additional motifs contribute to CD28-dependent T cell activation.

Materials and Methods

Mice

CD28-Y170F/AYAA knockin mice were generated as previously described, with the only difference being generation of a construct in which both the PYAP and YMNM sequences were mutated (9, 10). For wild-type mice,
a nonmutated CD28 allele was knocked in and backcrossed identically to the mutant alleles, as previously described (9). The correct sequence and germline transmission was verified at all stages by Southern blot analysis, direct sequencing, PCR analysis, and restriction digestion, as described for the single mutants. The mice were backcrossed into C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) and DO11.10 OVA-specific TCR-transgenic mice on the BALB/c background (14) (provided by K. Murphy, Washington University). All protocols were reviewed and approved by the Washington University School of Medicine Animal Studies Committee.

**Abs**

Anti-CD3ε (clone 145-2c11; Hm IgG) and all other fluorescently conjugated Abs used for staining were purchased from either eBioscience (San Diego, CA) or BD Biosciences (San Jose, CA). For T cell stimulations, anti-CD28 (clone 37.51; hamster IgG) was purchased from BD Biosciences. Anti-Bcl-xL (clone 2H12; mouse IgG) was purchased from Southern Biotech (Birmingham, AL).

**Proliferation and cytokine assays**

Splenocytes were isolated from 6-8-week old mice of each genotype and plated in quadruplicate wells at 1 × 10^6 cells/well in round-bottom 96-well tissue culture plates and stimulated as indicated for 48 h. Purified naïve CD4 T cells were isolated from DO11.10 mice by magnetic bead purification using a CD4^+/CD62L^+ MACS Magnetic Selection Kit (Miltenyi Biotec, Auburn CA). For proliferation, each well was pulsed with 1 μCi titrated thymidine overnight. For IL-2 and IFN-γ assays, all conditions were plated in triplicate. Supernatants were harvested at 48 h, and cytokines were determined by Cytokine Bead Array (BD Biosciences). All experiments were repeated a minimum of three times.

**Determination of Bcl-xL expression and viability**

Splenocytes were isolated and either cultured in media alone or stimulated with anti-CD3 (0.25 μg/ml) and anti-CD28 (1 μg/ml) for 48 h. Viability was determined by 7-aminoactinomycin D staining and flow cytometry. For determination of Bcl-xL expression, cells were stained with allophycocyanin-conjugated anti-CD4, followed by intracellular staining with PE-conjugated Bcl-xL, as previously described (10).

**Allergic airway inflammation**

Mice were immunized and challenged with OVA (Sigma-Aldrich, St. Louis, MO), as previously described (15). Samples were collected for analysis, as previously described, including bronchoalveolar lavage fluid for cell counts, differential analysis, and tissue histology (15). Spleens also were harvested, and frozen sections were prepared and stained with PNA-biotin and rat anti-mouse IgD, followed by detection with AP-streptavidin and goat anti-rat IgG (H+L)-HRP to identify GCs. Sections were examined for the number of GCs, and the mean number of GCs/10^4 field was determined.

**Results**

The CD28-Y170F/AYAA double-mutant mouse unmasks a role for the Y170 motif in CD28-dependent proliferation

Knockin mice in which both motifs were mutated (CD28-Y170F/AYAA) were generated as described for the mutant-CD28-Y170F and CD28-AYAA knockin mice (9, 10). The mutant mice were backcrossed to the C57BL/6J- or DO11.10-transgenic mice on the BALB/c background for 10 generations and then intercrossed to generate homozygous mice expressing two mutant alleles. The amino acid sequence for each strain and expression of CD28 on splenocytes and thymocytes are shown (Supplemental Fig. 1). Consistent with our prior reports, there was a slight decrease in the expression of CD28 on cells from the AYAA mutant mice. We detected no alteration in the number or percentage of CD4^+ or CD8^+ cells in the spleen or the thymus of the CD28-Y170F/AYAA mutant mice, nor did we detect reductions in the percentage of CD4/CD44^hi or CD8/CD44^hi T cells in any of the knockin mutants, although CD8/CD44^hi T cells were reduced in the CD28-knockout mice (data not shown, Supplemental Table I). As previously reported, the percentage of regulatory T cells was decreased in the CD28-AYAA mice (16) and were similar to the levels in the CD28-Y170F/AYAA mice.

We showed previously that T cells isolated from CD28-AYAA mice had a reduced CD28-dependent proliferative response compared with wild-type and CD28-Y170F cells; however, it remained consistently greater than that of CD28-deficient cells (9, 10). To determine whether the residual response was due to signaling initiated by the Y170 motif, we measured proliferation of cells from the CD28-Y170F/AYAA double mutant. The response of the double mutant to anti-CD3 alone was markedly impaired and was significantly less than that of the CD28-AYAA mutant (Fig. 1A). When the double-mutant cells were costimulated with anti-CD3 and anti-CD28, proliferation was augmented over anti-CD3 alone, although it

![Figure 1](http://www.jimmunol.org/)
remained less than that of CD28-AYAA–expressing cells (Fig. 1B, 1C). When stimulated with suboptimal levels of anti-CD28 in the presence of CTLA4 Ig to block endogenous co-stimulation, there was no detectable difference in proliferation between the CD28-AAYA and CD28-Y170F/AAYA cells (Fig. 1E), suggesting that masking of CD80/CD86 binding to the inhibitory counterreceptor, CTLA-4, may augment proliferation under these conditions, although this remains speculative. All genotypes proliferated comparably following activation with PMA and ionomycin, but the AAYA and Y170F/AAYA cells failed to respond to PMA plus anti-CD28 (Fig. 1D). Similar results were obtained using purified naive CD4+ T cells isolated from DO11.10 TCR-transgenic mice stimulated with OVA peptide (Fig. 1F), although the defect observed in the CD28-AAYA cells was less marked, suggesting there may be important differences depending on whether costimulation is provided by endogenous CD80/CD86 or by exogenous cross-linking Ab. These data demonstrate that the Y170 motif contributes to CD28-dependent proliferative responses and that this is unmasked when examined in the context of simultaneous mutation of the CD28-AAYA mutation. Furthermore, these data firmly establish that additional motifs can mediate CD28-dependent signaling.

In our previous work, we demonstrated that IL-2 secretion was markedly impaired in the CD28-AAYA mutant, yet it remained relatively intact in the CD28-Y170F mutant (9, 10). Because we observed an additive effect of each mutation on proliferation in the double mutant, we examined the effect on cytokine secretion. Consistent with our prior reports, both IL-2 and IFN-γ were reduced in the AAYA mutant, whereas the Y170F mutation alone resulted in little change (Fig. 2). However, in contrast to the results observed with proliferation, there was no greater impairment in the double mutant than observed with the CD28-AAYA single mutant. Similar results were found when cells were stimulated with lower doses of anti-CD28 (data not shown). Thus, the more severe proliferative defect observed with the CD28-Y170F/AAYA mutant cannot be accounted for by a comparable reduction in IL-2 secretion. Furthermore, because both the AAYA and double mutant secrete greater amounts of cytokines than do the CD28-deficient cells, additional motif(s) must be involved in regulating the overall levels of cytokine production.

Bcl-xL expression is coordinately regulated by the PYAP and Y170 motifs

CD28 regulates cell survival and induces the antiapoptotic protein Bcl-xL (17, 18). Previous work suggested that the Y170 motif was essential for this (19, 20). Thus, we examined cells from the double CD28-Y170F/AAYA mutant for overall viability and Bcl-xL expression (Fig. 3). Unstimulated cells of all genotypes had a similar percentage of cells expressing Bcl-xL; the level of expression, as measured by the mean fluorescence intensity, was also similar between strains. Following activation with anti-CD3/anti-CD28, only the CD28-deficient cells failed to increase the percentage of cells expressing Bcl-xL, and both the single and double mutants increased to the same extent as did wild-type cells. However, the amount of Bcl-xL per cell, as indicated by the mean fluorescence intensity, was slightly decreased in the CD28-Y170F and CD28-AAYA mutant cells. Importantly, this was decreased further in the Y170F/AAYA double mutant, demonstrating that both motifs are involved in CD28-dependent regulation of Bcl-xL and that compensatory signaling from the intact motif preserves expression in the single-mutant cells.

**In vivo responses of CD28-mutant mice**

Induction of allergic airway inflammation can be used to assess the role of CD28 in regulating an in vivo T cell–dependent immune response, because mice in which CD28 signaling is lacking fail to respond (21, 22). Both the CD28-AAYA and CD28-Y170F mutant mice develop airway inflammation in this model; however, GC formation is impaired in the CD28-AAYA and knockout mouse (9, 10). To determine whether there was synergy between the two motifs, we examined lung inflammation and GC formation in the double-knockin mouse. Wild-type mice mounted an eosinophilic inflammatory response in the lung and had GC formation in the spleen, whereas CD28-deficient mice did not develop lung inflammation or splenic GCs. All of the knockin mice developed...
lung inflammation to the same extent as did wild-type mice (Fig. 4A–C). However, GC formation was markedly impaired in the CD28-AYAA and double-mutant mice (Fig. 4D). These data demonstrate that, despite impairments in proliferation, cytokine secretion, and expression of Bcl-xL in vitro, mutation of either or both of the major signaling motifs in the tail of CD28 had little effect on development of an inflammatory response in the lung. In contrast, normal GC formation, which requires close T:B cell collaboration, remained dependent predominantly on the PYAP motif.

**Discussion**

In this study, we tested the hypothesis that the signaling through CD28 is mediated entirely by two cytoplasmic tail motifs. Using knockin mice expressing CD28, in which one or both of the motifs were mutated, we found minimal effect of the Y170F mutation when examined in isolation, whereas proliferation and cytokine secretion were reduced in the single AYAA mutant. However, when the Y170F and AYAA mutations were present simultaneously, proliferation and Bcl-xL expression were affected more severely than in the single-mutant cells, yet they remained reproducibly greater than those observed with CD28-deficient cells. Although there was a greater impairment of proliferation in the double mutant, IL-2 secretion was not different from what was observed in the single AYAA mutant and, therefore, cannot account for the greater proliferative defect.

We also examined the effect of the double mutation on an in vivo immune response. All genotypes, with the exception of the CD28-deficient mice, mounted an eosinophilic inflammatory cell response in the lung. In contrast, both the AYAA and Y170F/AYAA mutant mice had defects in GC formation, although there was no additive effect of the double mutant. Thus, similar to the in vitro results, these data support that additional motifs can mediate some biological effects of CD28 costimulation, whereas provision of T cell–derived signals to B cells are almost exclusively regulated by signals initiated by the distal proline motif. These results do not exclude the possibility that, under different experimental conditions, such as a less intense inflammatory stimulus, decreased Ag, or alternative adjuvant, defects in the response of the knockin mice might be unmasked.

These data support two major conclusions. First, that signaling initiated by the proximal tyrosine motif plays an important role in regulating CD28-dependent proliferation, despite the apparent lack of an effect of the single Y170F mutation. The experiments with the Y170F/AYAA double mutant effectively unmasked the contribution of the Y170 motif. Second, our data establish that additional motifs, either within the cytoplasmic tail or perhaps the transmembrane or extracellular domain, can contribute to CD28 signaling, because there remained residual CD28-dependent responses in the double-mutant cells. A limitation of the current study is that our data do not identify which motifs mediate this remaining signaling, nor do they establish that those motifs are active in the wild-type setting when the Y170 or PYAP motif is intact.

The readouts that we examined represent important outcomes of T cell activation and are the result of the integration of both TCR- and CD28-mediated signaling. One limitation of this study is that we have not provided data directly demonstrating which signaling pathways are impaired in the double mutant compared with each individual mutant. The adapter protein Grb2 can bind CD28 at both motifs, and binding to the YMNM motif is preserved when the tyrosine is mutated (5, 23, 24). Thus, the Y170F/AYAA mutant most likely retains some ability to bind Grb2, which may account for the residual signaling.

Our original study showing that the CD28-Y170F knockin mouse was phenotypically normal was surprising, given the large body of evidence supporting a role for this motif in CD28 signaling (3, 11–13, 25, 26). Early studies (3, 11) using cell lines demonstrated that mutation of the Y170 motif impaired IL-2 secretion. Subsequent studies (19, 20) in primary cells yielded differing results, with proliferation and IL-2 secretion being relatively spared, whereas induction of Bcl-xL was markedly reduced. However, our original examination of the Y170F knockin mouse revealed no decrease in Bcl-xL expression, despite defective PI3K recruitment and subsequent phosphorylation of protein kinase B (10). These observations led us to hypothesize that compensatory signaling pathways initiated by the PYAP motif accounted for the preserved expression of Bcl-xL in the Y170F mutant. This model is supported by the current data demonstrating that the double mutant has a much greater decrease in Bcl-xL expression than either single mutation alone.

The ability of motifs other than the Y170 or PYAP motif to support CD28 function is supported by a recent report by Pagán et al. (27), in which they measured naïve T cell expansion in vivo in the CD28-AYAA and CD28-Y170F mice. Using a peptide/MHC class II tetramer to track endogenous T cell numbers, they demonstrated that, although there was a marked defect in the expansion of CD28-deficient cells, this was not dependent on either motif. Immunization with peptide in the absence of any adjuvant unmasked a contribution by both the PYAP and Y170 motifs, although expansion still remained greater than in the CD28-deficient mice.
CD28 remains an attractive therapeutic target given the wide range of effects that costimulation has on T cell function (28). Global inhibition of CD28 function by blocking ligand engagement is an effective therapy for some chronic inflammatory diseases and prevention of transplant rejection (29, 30). However, these drugs may have mechanisms of action beyond preventing CD28-mediated costimulation (31–33). Our studies with the CD28-mutant knockin mice demonstrate that CD28 signaling is initiated by multiple motifs and that although some biologic outcomes can be mapped to one pathway, others involve a more complex interplay of signaling cascades. Additional studies will be needed to determine what accounts for the residual responses in the double-mutant mice, and whether these are important in normal CD28 functioning or whether they are only induced in the setting of the inactivation of the YMN M- and PYAP-signaling motifs. A greater understanding of these pathways may provide insights necessary to develop more targeted therapies that can selectively inhibit specific aspects of CD28-driven T cell functions while leaving desirable effects intact.

Acknowledgments

We thank the Alvin J. Siteman Cancer Center at Washington University School of Medicine and Barnes-Jewish Hospital (St. Louis, MO) for use of the Murine Embryonic Stem Cell Core, which provided embryonic stem cell injection service. The Siteman Cancer Center is supported in part by National Cancer Institute Cancer Center Support Grant P30 CA91842.

Disclosures

The authors have no financial conflicts of interest.

References


Downloaded from http://www.jimmunol.org/ by guest on April 14, 2017