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Vaccine Activation of the Nutrient Sensor GCN2 in Dendritic Cells Enhances Antigen Presentation

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Abstract

The yellow fever vaccine YF-17D is one of the most successful vaccines ever developed in humans. Despite its efficacy and widespread use in more than 600 million people, the mechanisms by which it stimulates protective immunity remain poorly understood. Recent studies using systems biology approaches in humans have revealed that YF-17D–induced early expression of general control nonderepressible 2 kinase (GCN2) in the blood strongly correlates with the magnitude of the later CD8⁺ T cell response. We demonstrate a key role for virus-induced GCN2 activation in programming dendritic cells to initiate autophagy and enhanced antigen presentation

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Supplementary Materials www.sciencemag.org/content/343/6168/313/suppl/DC1 Materials and Methods Figs. S1 to S27 Table S1 References

to both CD4⁺ and CD8⁺ T cells. These results reveal an unappreciated link between virus-induced integrated stress response in dendritic cells and the adaptive immune response.

Systems biological analysis of the immune response to the yellow fever vaccine in humans has identified early transcriptional signatures in the blood, which predict the magnitude of the later CD8⁺ T cell response to the vaccine (1). One of the genes contained within these signatures is the general control nonderepressible 2 kinase (GCN2)/EIF2AK4 (fig. S1), raising the possibility that GCN2 may be involved in the CD8⁺ T cell responses to YF-17D (1). GCN2 is a sensor of amino acid starvation in mammals and orchestrates the so-called integrated stress response (2–4). During amino acid starvation, GCN2 regulates protein synthesis through phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α) at Ser⁵¹ (5). The phosphorylation of eIF2 α attenuates assembly of the active preinitiation translational complex eIF2-GTP-tRNA^{Met} and polysome formation, resulting in stress granule formation (6). Recent work has also shown an antiviral effect of GCN2 against viruses (7–9) and in regulating the survival and proliferation of T cells (10, 11), but whether GCN2 mediates innate control of adaptive immunity is not clear. We have dissected the mechanisms through which GCN2 controls the antigen-specific T cell responses to YF-17D.

To determine whether YF-17D induces a stress response, we analyzed stress granule formation in human monocyte-derived dendritic cells (hmDCs) and baby hamster kidney (BHK) cells after YF-17D stimulation. Stress granules formed in response to YF-17D activation in vitro (Fig. 1, A and B). Further, Western blot analysis after culture with YF-17D revealed phosphorylation of eIF2 α in mouse bone marrow–derived dendritic cells (BMDCs) (Fig. 1C) and phosphorylation of GCN2 (Fig. 1D) and eIF2 α (Fig. 1D) in hmDCs after 1 hour. This rapid activation of GCN2 in vitro is different from the kinetics (d7) of the transcriptional signature observed in YF-17D vaccines (fig. S1), which is likely caused by an acute viremia in vivo.

YF-17D–induced eIF2 α phosphorylation was largely reduced by ultraviolet or heat inactivation of the virus, suggesting a requirement for live virus (fig. S2). Further, certain Toll-like receptor (TLR) ligands [CpG1826 and lipopolysaccharide (LPS), but not polyinosinic:polycytidylic acid] were capable of phosphorylating eIF2 α (fig. S3). Myeloid differentiation primary response gene 88 (MyD88) is a universal adaptor for TLR activation. Because YF-17D signals via multiple TLRs (12), we determined whether TLR signaling was necessary for phosphorylation of eIF2 α using *MyD88*^{−/−} mice. There was a partial reduction in eIF2 α phosphorylation in DCs from *MyD88*^{−/−} mice compared with wild-type mice (fig. S4), suggesting that YF-17D induces eIF2 α phosphorylation also independently of TLR.

Next, we analyzed whether GCN2 is necessary for the antigen-specific CD8⁺ T cell response to YF-17D using mice deficient in GCN2 (10). GCN2-deficient (*GCN2*^{−/−}) animals did not have any noticeable deficiencies in weight or appearance and had similar frequencies and numbers of immune-cell subsets (figs. S5 and S6). However, after vaccination with YF-17D the frequencies of interferon- γ (IFN- γ)–producing CD8⁺ T cells were substantially reduced in the liver and lung of *GCN2*^{−/−} mice (Fig. 2, A and B). In contrast to the robust responses in the liver and lung, responses were lower in the other organs but were nevertheless substantially reduced in *GCN2*^{−/−} mice, as assayed by a 4-day peptide

restimulation culture (figs. S7 and S8). Furthermore, the kinetics of the responses was similar in wild-type and *GCN2*^{-/-} mice (fig. S8). In addition, the CD4⁺ T cell responses were also markedly reduced in *GCN2*^{-/-} mice (Fig. 2C and fig. S7), demonstrating a role for GCN2 in the induction of T cell immunity to YF-17D.

To determine whether GCN2 deficiency in the hematopoietic compartment resulted in impaired immune responses, we generated bone marrow chimeras. There was a substantial defect in antigen-specific CD8⁺ T cell response in wild-type animals reconstituted with *GCN2*^{-/-} bone marrow, as compared with those reconstituted with wild-type bone marrow (Fig. 2, D and E, and fig. S9). To determine whether GCN2 expression in DCs is required for YF-17D-specific CD8⁺ T cell responses, we compared immunized *GCN2*^{fl/fl} CD11c-cre mice [in which *GCN2* was ablated in DCs (fig. S10)] and observed reduced frequencies of IFN- γ -producing CD8⁺ T cells in the lung and liver, as compared with that of littermate controls (Fig. 2, F and G).

To investigate the mechanism by which GCN2 expression in DCs controls T cell responses, we compared cytokine production by DCs from wild-type and *GCN2*^{-/-} mice, cultured in vitro with YF-17D. Induction of the inflammatory cytokines interleukin-6 (IL-6), tumor necrosis factor (TNF), IL-12, IL-1 β , or anti-inflammatory IL-10 (fig. S11) or antiviral IFN α (fig. S12) was unaffected by GCN2 deficiency. Furthermore, there was no difference in the induction of costimulatory molecules in vivo in response to vaccination with YF-17D (fig. S13) or in the uptake of soluble antigens (figs. S14 and S15).

Because GCN2 is a sensor of amino acid starvation (4), we determined whether YF-17D induced an amino acid starvation response in DCs. We used liquid chromatography/mass spectrometry (LC/MS) to analyze the intracellular concentration of free amino acids. Culture of hmDCs with YF-17D resulted in a rapid decrease of the intracellular concentration of free arginine and several other amino acids and a corresponding increase in citrulline (Fig. 3A). Arginine metabolism can lead to enhanced citrulline levels, a process catalyzed by nitric oxide synthase. Decreased intracellular concentrations of free amino acids, including arginine, leads to increased concentrations of uncharged transfer RNA molecules, which bind to the C terminus of GCN2 and induce its activation (13, 14). Consistent with changes in the concentrations of free amino acids, enzymes that are known to be important in amino acid metabolism were induced by YF-17D in DCs (fig. S16). Heat-killed or ultraviolet-irradiated YF-17D did not induce a noticeable reduction in amino acid concentrations, suggesting a requirement for live virus in this process.

Under conditions of amino acid deprivation, cells activate a homeostatic mechanism, called autophagy, that can effectively generate new amino acids and redirect the cell to use its limited amino acid supply specifically for the synthesis of essential proteins (15). GCN2 is a major sensor for amino acid deprivation in mammalian cells (4), and *GCN2*-deficient yeast are defective in induced autophagy (16). In this context, the expression of genes encoding autophagy-related proteins in peripheral blood mononuclear cells from humans vaccinated with YF-17D (1) correlated with the magnitude of the later CD8⁺ T cell responses (fig. S17). Consistent with this, culture of hmDCs (Fig. 3B) or mBMDCs (Fig. 3C) with YF-17D induced autophagy, as evidenced with visualization of autophagic vesicles (punctate LC3

staining). Furthermore, YF-17D induced autophagy in mBMDCs from LC3-GFP mice [in which the gene encoding green fluorescent protein (GFP) is fused with the gene encoding LC3 autophagosome marker, the mammalian homolog of Atg8 (17)] (fig. S18, A and B). To address whether induction of autophagy was dependent on GCN2, we examined YF-17D-induced autophagy in wild-type versus *GCN2*^{-/-} mice (Fig. 3, C to H). In *GCN2*^{-/-} mBMDCs, induction of autophagy was reduced as compared with that in DCs from wild-type mice, as assessed with immunofluorescence (Fig. 3, C and D) or Western blot (Fig. 3, E and F). In addition, reverse transcription polymerase chain reaction analyses revealed a marked reduction in the expression of genes encoding proteins involved in autophagy in *GCN2*^{-/-} DCs compared with wild-type DC stimulated from YF-17D (fig. S19). This was not due to baseline differences in autophagy proteins because basal expression of Atg5 and Atg7 in mBMDCs was identical in the absence of stimulation with YF-17D (fig. S20).

p62 and LC3-II accumulation are surrogate markers of autophagy because of their degradation after the fusion of the autophagosome to the lysosome (18). We therefore investigated the lysosomal turnover of endogenous p62 and LC3-II during virus-induced autophagy using either chloroquine (Fig. 3G) or the lysosomal protease inhibitors E64d plus pepstatin A, inhibitors of autophagy degradation (Fig. 3H). We observed greater accumulation of LC3-II (Fig. 3, G and H) and p62 (Fig. 3G) in the presence of these inhibitors in wild-type cells than in *GCN2*^{-/-} cells. Thus, these data demonstrate that induction of autophagy by YF-17D in DCs is controlled by GCN2.

Autophagy is known to affect antigen presentation by DCs (19, 20). Effective priming of CD8⁺ T cells by viral antigens involves the pathways that are active in both direct presentation of endogenous antigens and cross presentation of exogenous antigens (21, 22). Recent reports have implicated a role for autophagy in antigen presentation via both class II (23, 24) and class I (19, 25, 26). We thus hypothesized that *GCN2*^{-/-} DCs that are defective in autophagy may be impaired in their capacity to present antigens to T cells. In particular, viral antigens are cross-presented by DCs to CD8⁺ T cells (22), and given the impaired CD8⁺ T cell immunity to YF-17D, we determined whether cross-presentation of YF-17D antigens was impaired in *GCN2*^{-/-} mice. For this, lysates from YF-17D-Ova-infected BHK cells were cultured with BMDCs (precultured for 2 hours in low amino acid medium in order to induce the stress response) from wild-type or *GCN2*^{-/-} mice. The DCs were then washed and cultured with naïve, antigen-specific CD8⁺ T cell receptor transgenic T cells from OT-1 mice. The proliferation of antigen-specific CD8⁺ T cells was monitored by their ability to divide and incorporate thymidine. *GCN2*^{-/-} DCs had a lower ability to induce T cell proliferation in vitro, indicating a critical role for GCN2 in cross presentation (Fig. 4A). Antigen-specific responses were similar when transgenic OT1 T cells were cultured with either wild-type or *GCN2*^{-/-} BMDCs pulsed with SIINFEKL peptide (fig. S21), suggesting that DCs from *GCN2*^{-/-} DCs were not intrinsically defective at presenting peptide antigens to CD8⁺ T cells. Furthermore, direct antigen presentation of endogenous antigens was unaffected by GCN2 deficiency in DCs (fig. S22).

Atg5 and Atg7 are essential proteins required for autophagosome formation (27, 28). To examine the DC intrinsic effects of Atg-5 and Atg-7 deficiency, we used DC-specific conditional knockouts (fig. S23, A and B). BMDCs isolated from these mice or littermate

controls were initially cultured for 2 hours in low amino acid medium and then cocultured with lysates from BHK cells that had been previously infected with YF-17D-Ova (29). The BMDC loaded with YF-17D-Ova-infected BHK were then cultured with transgenic naïve OT-1 T cells, and proliferation was monitored by their ability to incorporate thymidine (Fig. 4, B and C). DCs from Atg-5- and Atg-7-deficient mice were impaired in their capacity to cross-present antigens (Fig. 4, B and C). Consistent with this, BMDCs from mice deficient in beclin-1—a protein necessary for Atg-5/Atg-7-dependent and independent-autophagy (30)—were impaired in their capacity to cross-present antigens from viral infected cells (fig. S24). A previous study demonstrates that Atg5 activity in DCs is not essential for cross-presentation of splenocytes coated with ovalbumin, in the absence of any viral stimulus or without any starvation (23). Consistent with our results, an analysis of the YF-17D-specific CD8⁺ and CD4⁺ T responses in the Atg5- and Atg7-conditional knockout mice revealed lower immune responses as compared with that of littermate controls (Fig. 4, D to I), and inhibiting autophagy with chloroquine resulted in lower cross-presentation (Fig. 4J). The results indicate that Atg5 and Atg7 expression on DCs is critical to prime CD8⁺ T and CD4⁺ T cells under starvation conditions.

GCN2-mediated induction of autophagy could operate in DCs (the “cross-presenting” cells) or in neighboring cells that were infected by YF-17D (“donor” cells) and taken up by DCs (fig. S25). Enhanced autophagy on dying donor cells taken up by antigen-presenting cells can stimulate efficient cross-presentation (25). To dissect the putative roles of GCN2 in the donor versus cross-presenting cell, wild-type or *GCN2*^{-/-} DCs were cultured with cell lysates from wild-type or *GCN2*^{-/-} mouse embryonic fibroblasts (MEFs) infected with YF-17D-Ova and assayed for their ability to prime OT-1 T cells (Fig. 4K). The Ova-specific responses were the highest when both the MEFs and DCs are of wild-type origin. These responses were lower when either the MEFs or DCs were from the *GCN2*^{-/-} mice. The lowest proliferative responses were seen in the cocultures that had MEFs and DCs from the *GCN2*^{-/-} mice indicating a key role for GCN2 on both the dying and the phagocytosing cell. The results indicate that Atg5 and Atg7 expression on DCs is critical to prime CD8 T cells under starvation conditions. One caveat of these experiments is the use of YF-17D encoding a surrogate antigen rather than viral antigens.

These data suggest that GCN2-mediated activation of autophagy programs DCs to cross-present viral antigen to CD8⁺ T cells. Components of the autophagy machinery including Atg5 and Atg7 have recently been implicated in recognition and clearance of dead cells by a process known as LC3-associated phagocytosis (LAP) (31). It is likely that depletion of free amino acids induced by the initiation of viral replication or an innate response triggered by live viral entry leads to the activation of GCN2, which then induces autophagy and antigen presentation. Consistent with this, inactivated virus was unable to induce amino acid depletion. The question of how autophagy intersects the cross-presentation pathway at the cell biological level (such as through degradation of autophagosomal cargo or LAP or both) remains to be determined.

Last, in order to determine whether this mechanism was more generally relevant to other viruses or microbes, we analyzed the role of GCN2 in eliciting immune responses to other pathogens (fig. S26). We saw a role for GCN2 in antigen-specific CD8 T cell responses to

the live attenuated influenza vaccine (fig. S27). We could not detect any differences between wild-type and *GCN2*^{-/-} mice in the immune responses to various other pathogens (fig. S26).

Taken together, these results demonstrate a role for GCN2 activation in DCs in modulating the adaptive immune response. This may have evolved as a mechanism of pathogen sensing that is capable of detecting “footprints” of a pathogen, such as depleted amino acids in a local micro-environment *in vivo*. Furthermore, such a mechanism may be relevant in the induction of immunity against tumors, in which rapidly proliferating tumor cells may cause a depletion of amino acids in the local microenvironment. In addition, this mechanism may affect immunity to vaccination during amino acid malnutrition in humans. Last, vaccine adjuvants that activate the GCN2-autophagy pathway may be useful in inducing robust T cell responses in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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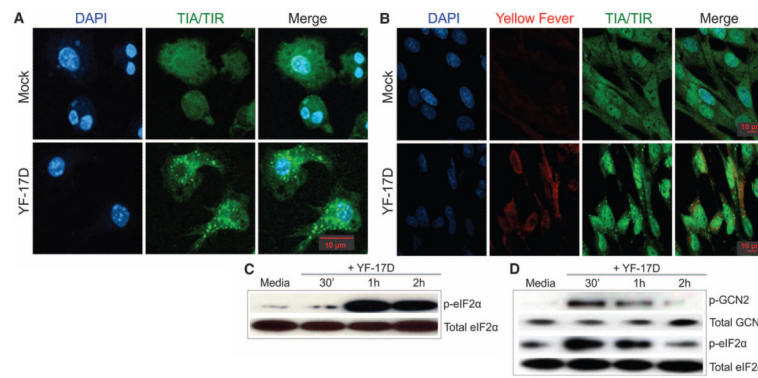


Fig. 1. YF-17D induces activation of the GCN2/eIF2 α -mediated stress response in DCs (A) hmDCs or (B) BHK cells were mock-treated or cultured with YF-17D (24 hours), fixed, stained with TIA/TIR and 4',6-diamidino-2-phenylindole to visualize stress granule formation. Mouse BMDCs (C) or hmDCs (D) were mock-treated or cultured with YF-17D for the times indicated. "Media" represents cells not exposed to the virus. (C and D) Phosphorylation of GCN2 and eIF2 α was assessed by means of Western blotting.

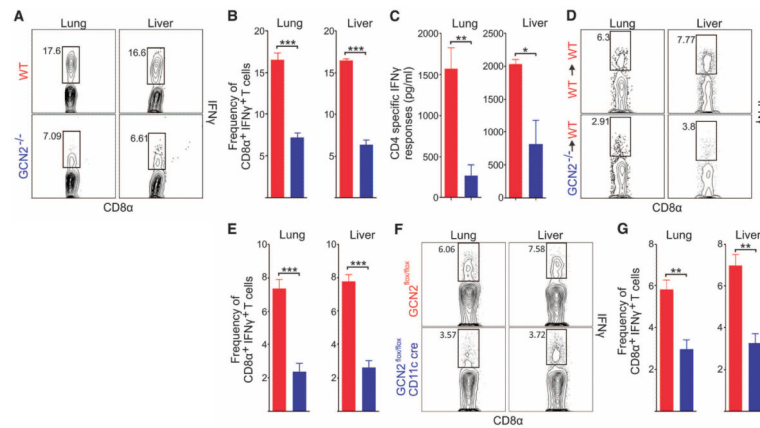


Fig. 2. Lack of GCN2 in dendritic cells leads to impaired CD8⁺ and CD4⁺ T cell responses to YF-17D

Mice were immunized subcutaneously with 2×10^6 plaque-forming units of YF-17D and euthanized on day 7. The magnitude of YF-17D-specific CD8 T cells was determined in the lung and liver with flow cytometry. (**A** and **B**) Representative fluorescence-activated cell sorting (FACS) plots (**A**) and frequencies (**B**) of CD8⁺ T cells producing IFN- γ in the lung and liver of wild-type versus *GCN2*^{-/-} mice. (**C**) Assessment of YF-17D-specific CD4⁺ T cells producing IFN- γ in culture after 4-day ex vivo restimulation with class II restricted peptides from YF-17D. (**D** and **E**) Representative FACS plots gated on CD8⁺ T cells producing IFN- γ (**D**) and frequencies (**E**) of CD8⁺ T cells producing IFN γ in the lung and liver of chimeric mice in which the hematopoietic compartment of wild-type mice was reconstituted with bone marrow from either wild-type or *GCN2*^{-/-} mice. (**F** and **G**) Representative FACS plots gated on CD8⁺ T cells producing IFN- γ in the lung and liver of *GCN2*^{fllox fllox} × *CD11c cre* versus littermate control mice. In (**E**), (**F**), and (**G**), red indicates wild type, and blue indicates *GCN2*^{-/-}. The graphs represent the averages of CD8⁺ IFN- γ -producing cells in the liver and lung in 5 mice per group. Data are representative of four independent experiments. ****P* < 0.0005; ***P* < 0.005; **P* < 0.05, calculated by Student's *t* test. Graphs show mean \pm SEM.

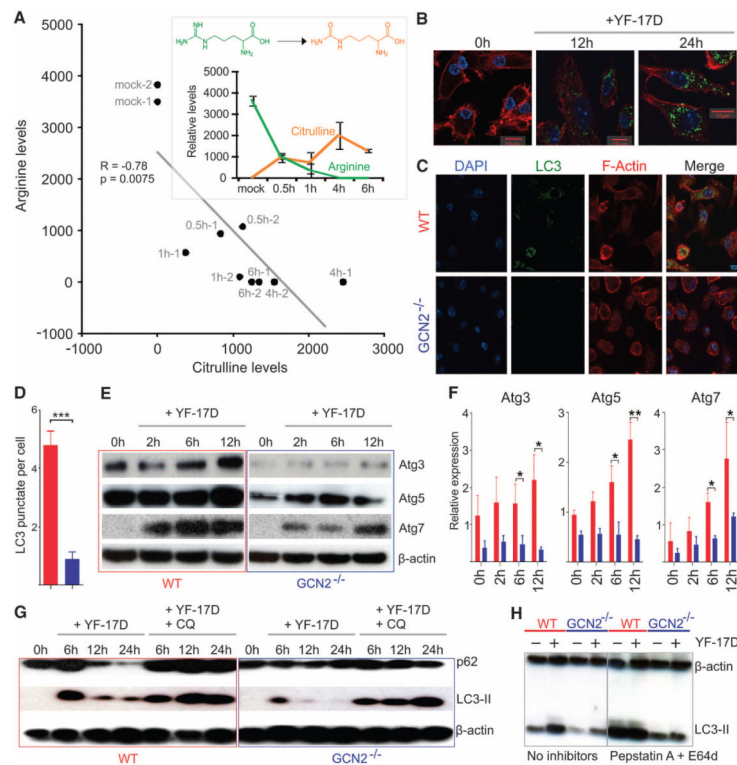


Fig. 3. YF-17D induces autophagy in dendritic cells via a mechanism dependent on GCN2
(A) Inverse correlation between the concentrations of free arginine and citrulline in hmDCs stimulated with YF-17D. Mock-treated DCs at 6 hours (without virus) are shown as controls. (Inset) Mean relative abundance \pm SD of arginine and citrulline. **(B)** Culture of hmDCs with YF-17D induces autophagy as visualized by means of confocal microscopy. **(C)** Comparison of autophagy (LC3 punctate staining) in mBMDCs from wild-type or *GCN2*^{-/-} mice, cultured in vitro with YF-17D for 6 hours. **(D)** Counts of LC3 granules per cell. **(E)** Comparison of the autophagy proteins in BMDC from wild-type or *GCN2*^{-/-} mice cultured in vitro with YF-17D. 0h represents 30 min after DCs are cultured in low volume of low fetal bovine serum medium with YF-17D. Basal levels of Atg5 and Atg7 in freshly isolated DC were similar in wild-type and *GCN2*^{-/-} mice (fig. S20). **(F)** Densitometric analysis of Western blots from three independent experiments. **(G)** Autophagy flux experiments depicting p62 and LC3II accumulation by chloroquine after culture with YF-17D. **(H)** Autophagy flux showing accumulation of LC3II 6 hours after YF-17D culture after treatment with lysosomal inhibitors (pepstatin and E64D). Data are representative of three independent experiments. **P* < 0.05; ***P* < 0.005, Student's *t* test. Error bars indicate mean \pm SEM

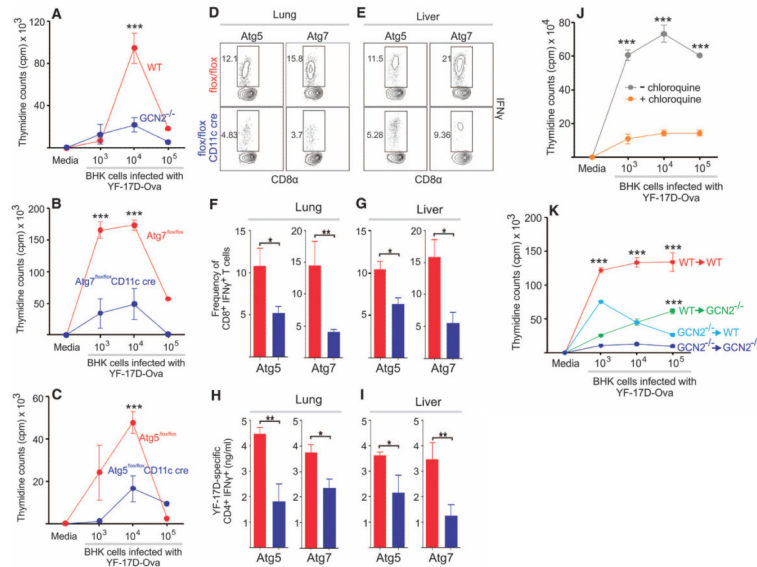


Fig. 4. YF-17D enhances the ability of DCs to cross-present antigens via a mechanism dependent on GCN2 and autophagy

BMDCs from wild-type mice, or *GCN2*^{-/-}, *Atg5*^{-/-}, or *Atg7*^{-/-} mice were cultured with lysates from BHK cells previously infected with YF-17D-Ova for 4 hours. The DCs were then cocultured with naïve OT-1 T cells. (A to C) Increased thymidine uptake showing increased proliferation by OT 1⁺ T cells stimulated by wild-type DCs as compared with (A) *GCN2*^{-/-}, (B) *Atg5*^{-/-}, and (C) *Atg7*^{-/-} DCs. (D to I) YF-17D-specific (D to G) CD8⁺ T cell responses and (H and I) CD4⁺ T cell responses in *Atg5*^{fl/fl}CD11c cre and *Atg7*^{fl/fl}CD11c cre mice compared with littermate controls 7 days after vaccination with YF-17D. (J) Inhibition of autophagy by chloroquine results in impaired cross-presentation. (K) MEFs from either wild-type or *GCN2*^{-/-} mice were first infected with YF-17D-Ova and then irradiated and then cocultured with either wild-type or *GCN2*^{-/-} BMDCs. Then OT-1 T cells were added, and their proliferation was evaluated 72 hours later. Representative of three individual experiments. ****P* < 0.0005; ***P* < 0.005; **P* < 0.05, calculated by Student's *t* test. Graphs show mean ± SEM.