

**The following resources related to this article are available online at [www.sciencemag.org](http://www.sciencemag.org) (this information is current as of July 10, 2009 ):**

**Updated information and services**, including high-resolution figures, can be found in the online version of this article at:

<http://www.sciencemag.org/cgi/content/full/325/5937/213>

**Supporting Online Material** can be found at:

<http://www.sciencemag.org/cgi/content/full/1172845/DC1>

This article **cites 21 articles**, 5 of which can be accessed for free:

<http://www.sciencemag.org/cgi/content/full/325/5937/213#otherarticles>

This article appears in the following **subject collections**:

Immunology

<http://www.sciencemag.org/cgi/collection/immunology>

Information about obtaining **reprints** of this article or about obtaining **permission to reproduce this article** in whole or in part can be found at:

<http://www.sciencemag.org/about/permissions.dtl>

context, it is interesting to note that the impairments after ablation of neurogenesis described here are parameter-sensitive (i.e., specific to conditions with a high premium on pattern separation), which may help to explain the variable and sometimes contradictory results from other neurogenesis-ablation studies in which this parameter was not explicitly considered (18, 24, 29, 30).

dnWNT mice with a ~50% decrease in neurogenesis had a similar pattern of impairment to that seen in the IR group in which neurogenesis was almost completely ablated, suggesting that there may be a critical threshold for the amount of neurogenesis that is behaviorally relevant. A level-dependent requirement of adult neurogenesis for hippocampus-dependent learning has also been reported in rats (24). In addition, our RAM task may be sufficiently challenging that even partial manipulation of newborn neuron numbers is adequate to impair performance.

The DG has been shown to be important for pattern separation, and our results show that adult neurogenesis appears to be important for the ability of the DG to perform that function optimally. It remains to be investigated whether immature neurons contribute to pattern separation directly or whether they contribute in more complex ways to a circuit necessary for normal DG function, as suggested by recent modeling studies (31), and whether the function of immature neurons is distinct from that of mature granule cells.

## References and Notes

- J. K. Leutgeb, S. Leutgeb, M.-B. Moser, E. I. Moser, *Science* **315**, 961 (2007).
- T. Nakashiba, J. Z. Young, T. J. McHugh, D. L. Buhl, S. Tonegawa, *Science* **319**, 1260 (2008); published online 24 January 2008 (10.1126/science.1151120).
- A. Bakker, C. B. Kirwan, M. Miller, C. E. L. Stark, *Science* **319**, 1640 (2008).
- D. Marr, *Philos. Trans. R. Soc. London Ser. B Biol. Sci.* **262**, 23 (1971).
- M. W. Jung, B. L. McNaughton, *Hippocampus* **3**, 165 (1993).
- D. G. Amaral, N. Ishizuka, B. Claiborne, *Prog. Brain Res.* **83**, 1 (1990).
- P. E. Gilbert, R. P. Kesner, I. Lee, *Hippocampus* **11**, 626 (2001).
- P. E. Gilbert, R. P. Kesner, W. E. DeCoteau, *J. Neurosci.* **18**, 804 (1998).
- M. R. Hunsaker, R. P. Kesner, *Hippocampus* **18**, 955 (2008).
- C. Zhao, W. Deng, F. H. Gage, *Cell* **132**, 645 (2008).
- H. van Praag *et al.*, *Nature* **415**, 1030 (2002).
- N. Toni *et al.*, *Nat. Neurosci.* **11**, 901 (2008).
- S. Jessberger, G. Kempermann, *Eur. J. Neurosci.* **18**, 2707 (2003).
- H. van Praag, B. R. Christie, T. J. Sejnowski, F. H. Gage, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 13427 (1999).
- E. Gould, A. Beylin, P. Tanapat, A. Reeves, T. J. Shors, *Nat. Neurosci.* **2**, 260 (1999).
- Materials and methods are available as supporting material on Science Online.
- L. Santarelli *et al.*, *Science* **301**, 805 (2003).
- J. S. Snyder, N. S. Hong, R. J. McDonald, J. M. Wojtowicz, *Neuroscience* **130**, 843 (2005).
- D. Bernstein *et al.*, *Pharmacol. Biochem. Behav.* **22**, 301 (1985).
- A. J. Morton, E. Skillings, T. J. Bussey, L. M. Saksida, *Nat. Methods* **3**, 767 (2006).
- J. C. Talpos, B. D. Winters, R. Dias, L. M. Saksida, T. J. Bussey, *Psychopharmacology (Berlin)* **205**, 157 (2009).
- S. M. McTighe, A. C. Mar, C. Romberg, T. J. Bussey, L. M. Saksida, *Neuroreport* **20**, 881 (2009).
- D. C. Lie *et al.*, *Nature* **437**, 1370 (2005).
- S. Jessberger *et al.*, *Learn. Mem.* **16**, 147 (2009).
- M. Fyhn, T. Hafting, A. Treves, M. B. Moser, E. I. Moser, *Nature* **446**, 190 (2007).
- S. Leutgeb *et al.*, *Science* **309**, 619 (2005).
- A. Vazdarjanova, J. F. Guzowski, *J. Neurosci.* **24**, 6489 (2004).
- P. E. Gilbert, R. P. Kesner, *Behav. Neurosci.* **117**, 1385 (2003).
- C. L. Zhang, Y. Zou, W. He, F. H. Gage, R. M. Evans, *Nature* **451**, 1004 (2008).
- J. M. Wojtowicz, M. L. Askew, G. Winocur, *Eur. J. Neurosci.* **27**, 1494 (2008).
- J. B. Aimone, J. Wiles, F. H. Gage, *Neuron* **61**, 187 (2009).
- We thank M. J. Armstrong and J. B. Aimone for useful input and discussions, M. L. Gage for editorial comments, and J. Simon for assistance with figures. This work was funded in part by James S. McDonnell, Lookout, and Mather's Foundations; the Kavli Institute for Brain and Mind; and NIH (grant NS-050217) (F.H.G.), and also by the NIHR Biomedical Research Centre Award to the University of Cambridge and generous donations from patients and families to the Huntington's and Parkinson's Disease Research Clinics at the Brain Repair Centre, University of Cambridge (R.A.B.). This work was also supported in part by MaxNetAging and NCCR Neural Plasticity and Repair Grants (S.J.) and Marshall's and Jack Kent Cooke Foundation scholarships (C.D.C.).

## Supporting Online Material

www.sciencemag.org/cgi/content/full/325/5937/210/DC1

Materials and Methods

Figs. S1 to S5

References

9 March 2009; accepted 26 May 2009

10.1126/science.1173215

# IRAP Identifies an Endosomal Compartment Required for MHC Class I Cross-Presentation

Loredana Saveanu,<sup>1</sup> Oliver Carroll,<sup>1</sup> Mirjana Weimershaus,<sup>1</sup> Pierre Guernonprez,<sup>2</sup> Elke Firat,<sup>3</sup> Vivian Lindo,<sup>4</sup> Fiona Greer,<sup>4</sup> Jean Davoust,<sup>1</sup> Roland Kratzer,<sup>1</sup> Susanna R. Keller,<sup>5\*</sup> Gabriele Niedermann,<sup>3\*</sup> Peter van Endert<sup>1†</sup>

Major histocompatibility complex (MHC) class I molecules present peptides, produced through cytosolic proteasomal degradation of cellular proteins, to cytotoxic T lymphocytes. In dendritic cells, the peptides can also be derived from internalized antigens through a process known as cross-presentation. The cellular compartments involved in cross-presentation remain poorly defined. We found a role for peptide trimming by insulin-regulated aminopeptidase (IRAP) in cross-presentation. In human dendritic cells, IRAP was localized to a Rab14<sup>+</sup> endosomal storage compartment in which it interacted with MHC class I molecules. IRAP deficiency compromised cross-presentation in vitro and in vivo but did not affect endogenous presentation. We propose the existence of two pathways for proteasome-dependent cross-presentation in which final peptide trimming involves IRAP in endosomes and involves the related aminopeptidases in the endoplasmic reticulum.

Peptide ligands for MHC class I molecules are produced by intracellular proteases (1). Initial antigen degradation by cytosolic proteasome complexes is frequently followed by N-terminal peptide trimming, which can occur in the cytosol and by endoplasmic reticulum (ER) aminopeptidases (ERAPs) (2). Peptides are transported into the ER by the transporter associated with antigen processing (TAP) for loading of newly

synthesized MHC class I molecules. Loading of MHC class I molecules with internalized, cross-presented antigens in dendritic cells (DCs) is thought to play an important role in priming of CD8<sup>+</sup> T cell responses to pathogens and tumors, as well as in immune tolerance to self.

While screening crude microsome lysates for peptidases involved in N-terminal trimming of human leukocyte antigen (HLA) class I ligands,

we identified insulin-regulated aminopeptidase (IRAP). IRAP was detected as an interferon  $\gamma$  (IFN- $\gamma$ )-induced activity trimming a fluorogenic Leu-aminomethyl coumarin (AMC) substrate in anion exchange chromatography (Fig. 1A) (3). The peak containing IRAP also trimmed a precursor of the HLA-A2-restricted epitope SLYNTVATL (4, 5). IRAP is a ubiquitous zinc-dependent aminopeptidase closely related to ERAP1 and ERAP2 (6). IRAP localizes to regulated endosomal storage compartments in adipocytes and muscle cells together with the glucose transporter Glut4; these compartments are termed Glut4 storage vesicles (GSVs) (7). Signaling through the insulin or immunoglobulin E (IgE) receptors induces rapid translocation of ~50% of IRAP to the cell surface (7, 8). The function of the compartment storing IRAP in other cell types, such as DCs, remains unknown.

To evaluate whether IRAP qualifies as a trimming aminopeptidase, we tested its sub-

<sup>1</sup>INSERM, U580, 75015 Paris, France; Université Paris Descartes, Faculté de Médecine René Descartes, 75015 Paris, France.

<sup>2</sup>INSERM, U653, 75006 Paris, France; Institut Curie, Centre de Recherche, 75006 Paris, France. <sup>3</sup>Clinic for Radiotherapy, University Hospital of Freiburg, 79106 Freiburg, Germany.

<sup>4</sup>M-SCAN Ltd., Wokingham, Berkshire RG41 2TZ, UK. <sup>5</sup>Division of Endocrinology, Department of Internal Medicine, University of Virginia, Charlottesville, VA 22908, USA.

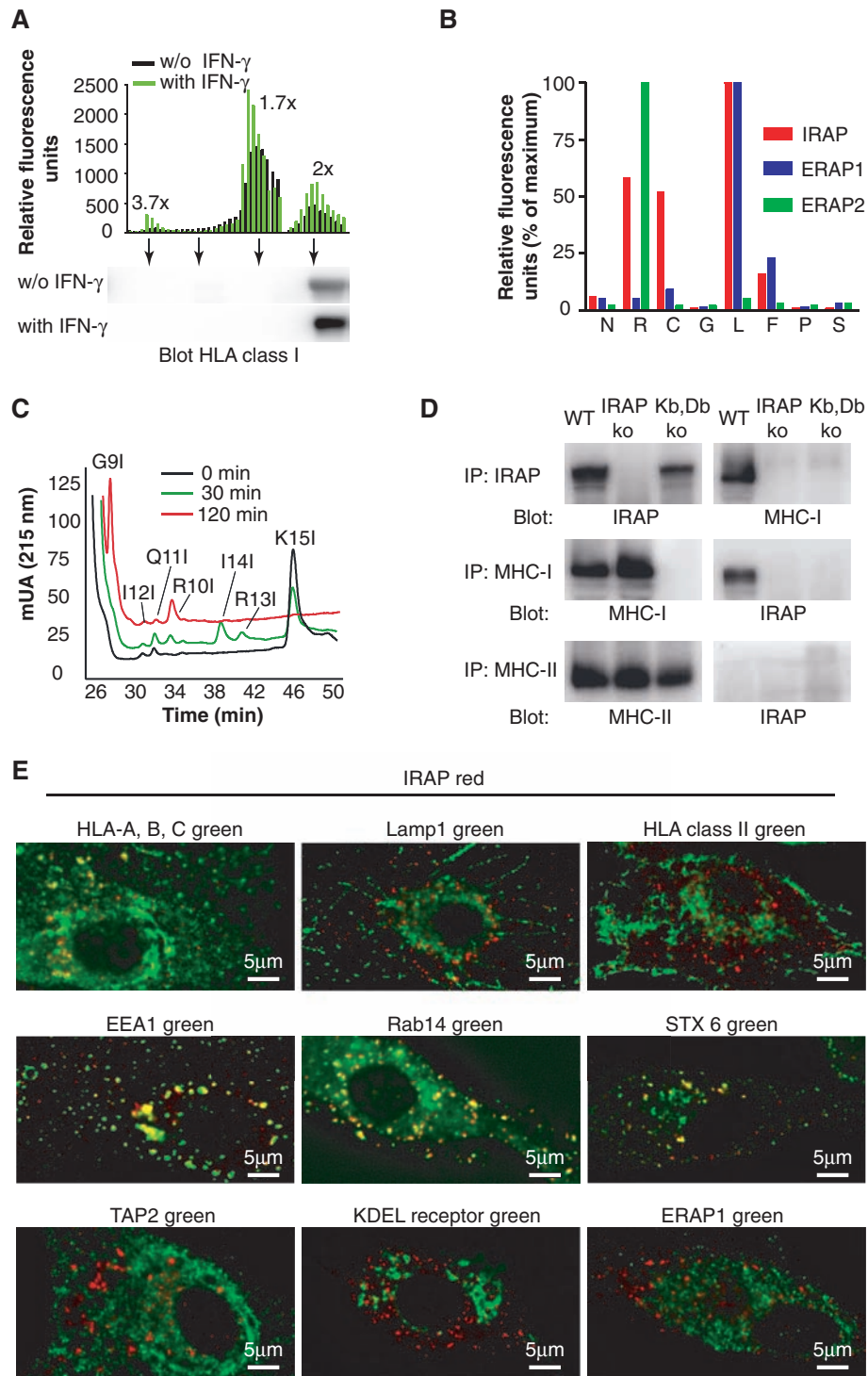
\*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: peter.van-endert@inserm.fr

strate specificity and interaction with MHC class I molecules. Human IRAP displayed a broader specificity toward fluorogenic substrates than did its ER-resident relatives (Fig. 1B). IRAP also efficiently converted the 15-nucleotide oligomer epitope precursor K15I to the minimal epitope G9I, which in the ER requires the concerted action of ERAP1 and ERAP2 (4) (Fig. 1C). The amount of IRAP eluted was doubled by IFN- $\gamma$  incubation of HeLa cells (Fig. 1A), although IRAP mRNA levels were not (fig. S1). Because IRAP eluted in a fraction also containing MHC class I molecules (Fig. 1A), increased recovery of IRAP activity could have resulted from its association with IFN- $\gamma$ -induced MHC molecules. A fraction of cellular IRAP was coprecipitated with HLA class I molecules from bone marrow-derived DCs (BMDCs) (Fig. 1D), compatible with a role for IRAP in MHC class I antigen presentation.

Because IRAP resides in endocytic vesicles (7), we considered that it may be involved in the MHC class I cross-presentation of exogenous antigens internalized by DCs through phagocytosis or receptor-mediated endocytosis (9). Although cross-presented antigens are believed to be processed mainly by factors also involved in the endogenous pathway, such as TAP, Sec61, or ERAP1 (10), the intracellular routes mediating the junction between the endocytic pathway and the ER have been difficult to decipher (9). In human myeloid DCs and murine BMDCs, ~25% of IRAP colocalized with HLA class I molecules (Fig. 1E and fig. S2), whereas no colocalization was observed with endolysosomal proteins (HLA class II and Lamp-1). In the absence of DC phagocytosis, we could not detect colocalization with three ER-resident proteins: KDEL receptor, ERAP1, and TAP2. This was not due to a failure of antibodies to recognize ER-resident IRAP, because strong colocalization with two ER markers of a hemagglutinin (HA)-tagged IRAP variant carrying a KDEL sequence was readily detectable, whereas ER colocalization was completely absent for HA-tagged IRAP lacking a KDEL sequence (fig. S3). Thus, newly synthesized IRAP molecules must exit rapidly from the ER. However, IRAP showed considerable colocalization with syntaxin 6 (STX6; 51%), a Q-SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment) known to stain GSVs (11). IRAP colocalized most strongly (76%) with Rab14, which may play a role in preventing phagosome fusion with lysosomes in macrophages (12, 13). Similar data were obtained in murine BMDCs (fig. S4), where IRAP also showed considerable colocalization with the mannose receptor (MR) (57%), reported to mediate efficient cross-presentation of soluble antigen (14).

During phagocytosis, IRAP was strongly enriched in purified early phagosomes but not in late phagosomes, whereas ERAP was not enriched in phagosomes (Fig. 2A), consistent with a recent proteomics analysis of phagosomes (15). In DCs phagocytosing yeast cells, IRAP colocalized preferentially with MHC class I molecules



**Fig. 1.** Identification of an MHC class I-associated aminopeptidase. (A) Hydrolysis of Leu-AMC by fractionated crude microsomal proteins from untreated or IFN- $\gamma$ -treated HeLa cells. Numbers above peaks indicate relative induction of Leu-AMC hydrolysis by IFN- $\gamma$  incubation. MHC class I was detected exclusively in peak 4 by immunoblot analysis; ERAP1 and ERAP2 elute exclusively in peak 1 under identical chromatographic conditions (4). (B) Recombinant human IRAP, ERAP1, and ERAP2 were tested for aminoacyl-AMC hydrolysis. For each enzyme, results are expressed as percent of maximum hydrolysis set at 100. (C) Peptide K15I (KIRIQRGPGRAVFI) (5) was digested with recombinant IRAP. Degradation products are designated by the N-terminal residue followed by peptide length and C-terminal residue. (D) IRAP, MHC class I, and MHC class II molecules were immunoprecipitated (IP) from BMDCs lysed with a mild detergent. Precipitates were split at a ratio of 1:9 for detection of directly precipitated and coprecipitated proteins, and analyzed by immunoblot as indicated. Control DCs were from K<sup>b</sup>D<sup>b</sup> $\beta$ 2m knockout (ko), HLA-B7 transgenic mice; WT, wild type. (E) Subcellular localization of IRAP (red) in human myeloid DCs.

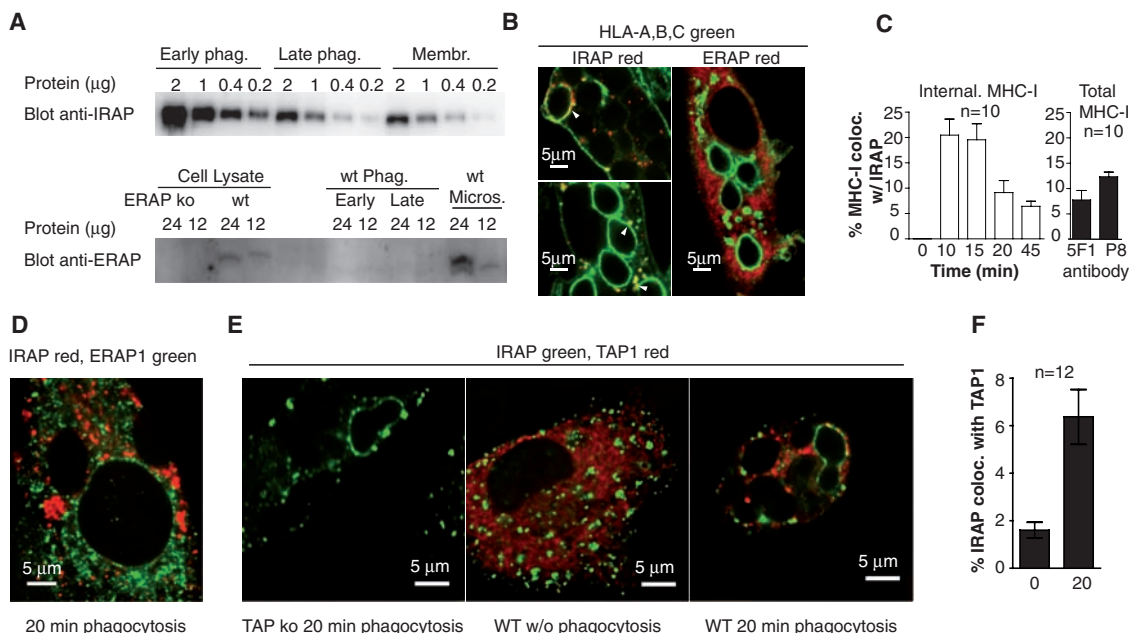
internalized from the surface in vesicles adjacent to phagosomes, or in phagosomal membranes (Fig. 2, B and C, and fig. S5). Internalized MHC

class I molecules could not be detected in late Lamp1<sup>+</sup> phagosomes (fig. S5). ERAP did not colocalize with internalized MHC class I mole-

cules (<0.5%; Fig. 2B). This suggests that internalized MHC class I molecules may traffic together with IRAP from phagosomes to GSV-

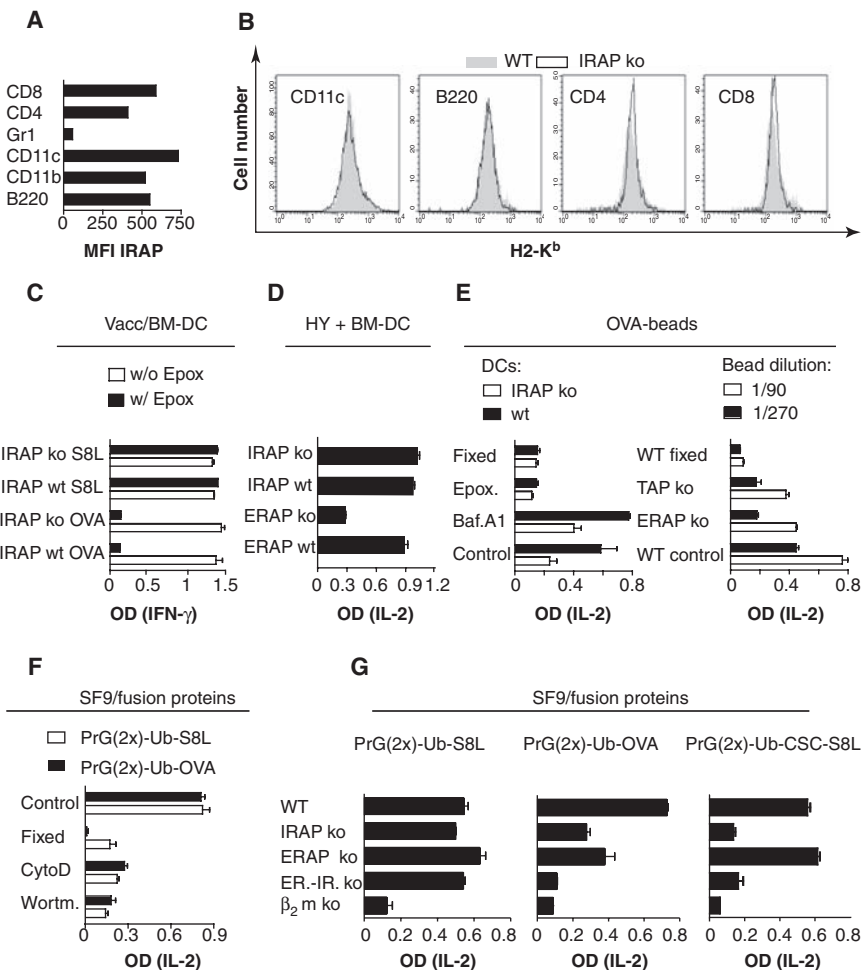
**Fig. 2.** IRAP, but not ERAP, is recruited to phagosomes.

(A) Serial dilutions of membranes from early (20 min) and late (2 hours) BMDC phagosomes were examined for IRAP and ERAP by immunoblot. Total cellular proteins or membrane proteins, and crude microsomes served as controls. One of two (ERAP blot) or four (IRAP blot) similar experiments is shown. (B) Human myeloid DCs were stained after 20 min of yeast cell phagocytosis for IRAP, ERAP, and HLA-A, B, and C. (C) BMDCs were stained for cell surface K<sup>b</sup> molecules, fed yeast cells, and analyzed for colocalization of IRAP with K<sup>b</sup> after different intervals. The right panel shows the percent colocalization (mean ± SD) of total cellular MHC class I with IRAP after 15 min. (D) Human myeloid DCs were stained for IRAP and ERAP. (E) Murine BMDCs, before or 20 min after phagocytosis of yeast cells, were stained with antibodies to IRAP and mouse TAP1. (F) The percentage of IRAP colocalizing with TAP1 was calculated using correlation maps.

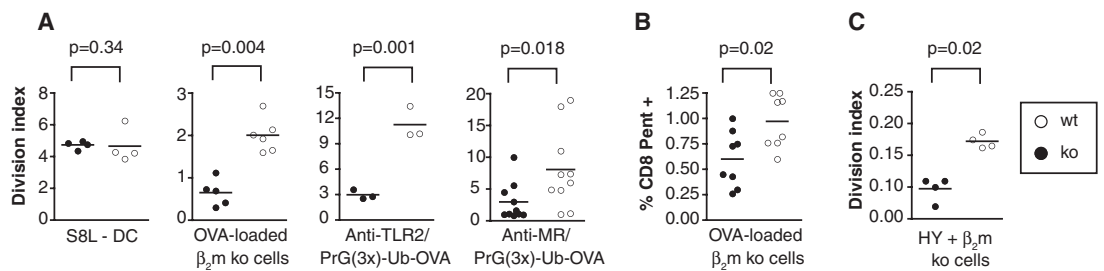


**Fig. 3.** IRAP is required for cross-presentation in vitro.

(A) Fluorescence-activated cell sorter analysis of intracellular IRAP expression in mouse splenocyte subpopulations. (B) Expression of H2-K<sup>b</sup> on splenic DCs (CD11c<sup>+</sup>), B cells (B220<sup>+</sup>), helper (CD4<sup>+</sup>), and killer (CD8<sup>+</sup>) T cells. (C) Generation of S8L in the endogenous pathway by vaccinia-infected DCs. The response of OT-I effector T cells was measured by IFN- $\gamma$  enzyme-linked immunosorbent assay. Epox, epoxomicin (proteasome inhibitor); wt, wild type. (D) Endogenous presentation of the SMCY male antigen by DCs to HY CD8<sup>+</sup> T cells. (E) In vitro cross-priming of OT-I cells by DCs incubated with OVA-coated beads. Baf.A1, bafilomycin A1. (F) In vitro cross-priming of OT-I cells with necrotic insect cells containing fusion proteins. CytoD, cytochalasin D; Wortm., wortmannin. (G) DCs were incubated with necrotic insect cells expressing fusion proteins comprising OVA, S8L, or S8L with an N-terminal CSC extension. The experiments shown correspond to one of two [(A), (D), (F), and (G)], three [(C) and (E)], or five (B) experiments. Means + SD are shown in (C) to (G). Splenic DCs were used in (E), BMDCs in all others.



**Fig. 4.** IRAP deficiency compromises *in vivo* cross-priming. **(A)** Mice were injected with CFSE-labeled OT-I cells, followed 24 hours later by injection of antigen. Three days later, OT-I proliferation (expressed as divisions per precursor OT-I cell) was examined by flow cytometry. Antigens were S8L-pulsed BMDCs,  $\beta_2m$  knockout splenocytes electroporated with OVA, or OVA fusion proteins targeted for internalization through TLR2 or the MR. **(B)** Mice were injected with OVA-loaded  $\beta_2m$  knockout splenocytes. Ten days later, priming of endogenous CD8<sup>+</sup> T cells was measured by staining with a K<sup>b</sup>/S8L pentamer. **(C)** SV129 mice were injected with CFSE-labeled HY T cells, followed 24 hours later by injection of male MHC-unmatched (Balb/c) splenocytes.



like vesicles before phagosome fusion with lysosomes takes place. DC activation by pathogen compounds induces TAP recruitment to MR<sup>+</sup> endosomes (14). Phagocytic DC activity did not increase IRAP colocalization with ERAP (Fig. 2D). In contrast, colocalization of IRAP with TAP increased from <2% in resting murine BMDCs to 6.5% (Fig. 2, E and F). Thus, ER-phagosome or endosome fusion events may selectively deliver TAP but not ERAP1 to IRAP<sup>+</sup> vesicles.

To examine a potential role of IRAP in antigen presentation, we studied previously generated IRAP knockout mice (8, 16). Deficiency for IRAP, which is normally expressed by all mouse splenocyte subpopulations except granulocytes (Fig. 3A), did not affect MHC class I expression by splenic lymphocytes including CD11c<sup>hi</sup> DCs (Fig. 3B), which also matured normally upon LPS stimulation (fig. S6). BMDCs from IRAP knockout mice expressing full-length ovalbumin (OVA) or the preprocessed K<sup>b</sup>-restricted OVA epitope S8L (SIINFEKL, OVA<sub>257-64</sub>), or pulsed with the synthetic epitope, presented epitope S8L normally to specific OT-I CD8<sup>+</sup> T cells (17) (Fig. 3C and fig. S7). Thus, production of epitope S8L in the endogenous processing pathway is proteasome-dependent (Fig. 3C) and ERAP-dependent (18), but does not require IRAP. Endogenous presentation of the male antigen SMCY (19) also required expression of ERAP but not IRAP by DCs (Fig. 3D). These results provide strong evidence against involvement of IRAP in the endogenous MHC class I processing pathway.

To study the impact of IRAP deficiency on cross-presentation, we first examined the presentation of OVA-coated beads to OT-I cells. The efficiency of bead phagocytosis by BMDCs was not affected by IRAP deficiency (fig. S8). Cross-presentation of beads was abolished by two proteasome inhibitors but was not affected, or even increased, by two inhibitors of lysosomal proteases (Fig. 3E and fig. S9). Cross-presentation also was compromised by TAP deficiency (Fig. 3E). Thus, OVA internalized by phagocytosis was shuttled into the pathway involving antigen degradation in the cytosol, but not into the vacuolar pathway involving acid lysosomal proteases (20, 21). Cross-presentation of particulate OVA was reduced by ERAP deficiency (Fig. 3E) (18, 22). IRAP deficiency resulted in an at least equal reduction of

presentation by 50 to 70% (Fig. 3E). This result suggests that precursors of epitope S8L could be trimmed both by an ER aminopeptidase and an endosomal aminopeptidase.

Next, we studied cross-presentation of necrotic insect cells expressing fusion proteins consisting of two or three Ig-binding domains derived from protein G (PrG), ubiquitin (Ub), and OVA or S8L. In the cytosol, OVA or S8L antigen is expected to be removed from these proteins by deubiquitinating enzymes. Cross-priming of OT-I cells required actin and phosphatidylinositol 3-kinase-dependent uptake of antigenic material by live DCs, demonstrating the absence of free peptide in the material (Fig. 3F). Generation of S8L from a phagocytosed fusion protein containing the preprocessed epitope not requiring trimming was not compromised in IRAP or ERAP single- or double-deficient DCs (Fig. 3G). However, absence of IRAP or ERAP reduced generation of S8L from a fusion protein containing full-length OVA. Absence of both peptidases had an additive effect (Fig. 3G), which suggests that the two enzymes may act in independent pathways. S8L generation from a precursor peptide extended by a sequence adapted to the cleavage specificity of IRAP but not ERAP [PrG(2x)-Ub-CSC-S8L] was reduced by >75% in IRAP-deficient DCs but was not affected by ERAP deficiency (Fig. 3G). Thus, IRAP acts as an epitope-trimming peptidase in an endosomal compartment.

Finally, we examined cross-priming of carboxyfluorescein succinimidyl ester (CFSE)-labeled, adoptively transferred naïve OT-I T cells *in vivo* (Fig. 4). IRAP deficiency did not affect recovery from lymph nodes and spleens of transferred OT-I cells (fig. S10), nor did it alter proliferation of transferred OT-I cells in response to S8L-pulsed DCs (Fig. 4A). In contrast, cross-presentation of cell-associated OVA was reduced (Fig. 4A and fig. S11). Next, we immunoaffinity-purified the fusion protein PrG(3x)-Ub-OVA and targeted it *in vivo* to cells expressing Toll-like receptor 2 (TLR2) or the MR via binding of its PrG domains to specific antibodies. IRAP deficiency resulted in reduced cross-presentation of receptor-targeted fusion protein (Fig. 4A and fig. S11). Moreover, IRAP deficiency reduced cross-priming of endogenous CD8<sup>+</sup> T cells by cell-associated OVA, as revealed by analysis of splenocytes from primed

mice with K<sup>b</sup>/S8L pentamers (Fig. 4B). Cross-presentation of a second antigen, cell-associated SMCY male antigen, was also compromised in IRAP knockout mice (Fig. 4C).

Our findings indicate that the final proteolytic trimming of cross-presented antigens can occur in an endosomal DC compartment sharing several markers associated with regulated endosomal storage vesicles. Recruitment of Rab14 may reduce routing of antigens into an acid lysosomal environment known to be detrimental for cross-presentation (23). Physical association of IRAP with abundant, presumably internalized class I molecules may favor a direct linkage between peptide trimming and MHC class I loading. Cross-presentation of antigens processed in an IRAP-dependent manner required active proteasome but not lysosomal proteases, which suggests that this pathway implicates cytosolic antigen degradation followed by peptide transport into IRAP<sup>+</sup> endosomes by TAP recruited upon phagocytosis. However, considering the relatively efficient TAP-independent cross-presentation of S8L (Fig. 3E), the existence of a pathway implicating IRAP together with an alternative endosomal peptide transporter, or together with a role for the proteasome unrelated to antigen degradation, cannot be ruled out entirely.

We found that both IRAP and ERAP are implicated in cross-presentation. Considering the functional redundancy and the complete absence of colocalization between the enzymes, the existence of two parallel proteasome-dependent cross-presentation pathways is the most plausible explanation (fig. S12). According to our model, MHC class I molecules can be loaded with cross-presented peptides in three intracellular compartments: endosomes, ER, and lysosomes/vacuoles.

**References and Notes**

1. N. Shastri, S. Cardinaud, S. R. Schwab, T. Serwold, J. Kunisawa, *Immunol. Rev.* **207**, 31 (2005).
2. L. Saveanu, O. Carroll, Y. Hassainya, P. van Endert, *Immunol. Rev.* **207**, 42 (2005).
3. See supporting material on *Science* Online.
4. L. Saveanu *et al.*, *Nat. Immunol.* **6**, 689 (2005).
5. Single-letter abbreviations for amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.
6. M. Tsujimoto, A. Hattori, *Biochim. Biophys. Acta* **1751**, 9 (2005).
7. S. R. Keller, *Front. Biosci.* **8**, s410 (2003).

8. M. G. Wallis, M. F. Lankford, S. R. Keller, *Am. J. Physiol.* **293**, E1092 (2007).
9. N. Monu, E. S. Trombetta, *Curr. Opin. Immunol.* **19**, 66 (2007).
10. A. L. Ackerman, A. Giardini, P. Cresswell, *Immunity* **25**, 607 (2006).
11. A. M. Shewan *et al.*, *Mol. Biol. Cell* **14**, 973 (2003).
12. C. Kuijl *et al.*, *Nature* **450**, 725 (2007).
13. G. B. Kyei *et al.*, *EMBO J.* **25**, 5250 (2006).
14. S. Burgdorf, C. Scholz, A. Kautz, R. Tampe, C. Kurts, *Nat. Immunol.* **9**, 558 (2008).
15. L. D. Rogers, L. J. Foster, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 18520 (2007).
16. S. R. Keller, A. C. Davis, K. B. Clairmont, *J. Biol. Chem.* **277**, 17677 (2002).
17. K. A. Hogquist *et al.*, *Cell* **76**, 17 (1994).
18. E. Firat *et al.*, *J. Immunol.* **178**, 2241 (2007).
19. P. Kisielow, H. Bluthmann, U. D. Staerz, M. Steinmetz, H. von Boehmer, *Nature* **333**, 742 (1988).
20. L. Ramachandra *et al.*, *Immunity Rev.* **168**, 217 (1999).
21. L. Shen, L. J. Sigal, M. Boes, K. L. Rock, *Immunity* **21**, 155 (2004).
22. J. Yan *et al.*, *J. Exp. Med.* **203**, 647 (2006).
23. A. Savina *et al.*, *Cell* **126**, 205 (2006).
24. We thank Metabolex Inc. for an anti-IRAP serum, N. Shastri for an anti-ERAP serum, B. Rocha for HY mice, O. Lantz for TAP knock-out mice, L. Chatenoud for OT-II mice, B. Fouquet and N. Merzougui for technical help, and A. M. Lennon-Dumesnil for critical reading of the manuscript. Supported by grant PROTARVAC of the

European Commission (P.V.E., F.G., G.N.), by INSERM fellowships (O.C., R.K.), and by grants from the Deutsche Forschungsgemeinschaft and from the Medical Faculty of the University of Freiburg (G.N.).

### Supporting Online Material

www.sciencemag.org/cgi/content/full/1172845/DC1

Materials and Methods

Figs. S1 to S12

References

27 February 2009; accepted 22 May 2009

Published online 4 June 2009;

10.1126/science.1172845

Include this information when citing this paper.

# Hematopoietic Cytokines Can Instruct Lineage Choice

Michael A. Rieger, Philipp S. Hoppe, Benjamin M. Smejkal, Andrea C. Eitelhuber, Timm Schroeder\*

The constant regeneration of the blood system during hematopoiesis requires tightly controlled lineage decisions of hematopoietic progenitor cells (HPCs). Because of technical limitations, differentiation of individual HPCs could not previously be analyzed continuously. It was therefore disputed whether cell-extrinsic cytokines can instruct HPC lineage choice or only allow survival of cells that are already lineage-restricted. Here, we used bioimaging approaches that allow the continuous long-term observation of individual differentiating mouse HPCs. We demonstrate that the physiological cytokines, macrophage colony-stimulating factor and granulocyte colony-stimulating factor, can instruct hematopoietic lineage choice.

All blood cells are generated from progenitor cells with more than one lineage potential (hematopoietic progenitor cells, HPCs). Hematopoiesis depends on tightly controlled lineage choice. Cytokines are necessary and sufficient for the production of specific mature blood cell types (1). However, despite decades of research, it is disputed whether cytokines instruct HPCs to differentiate into a specific lineage (2). Alternatively, cytokines may simply allow the survival or proliferation of cells that had already independently decided for this lineage. The cytokines' function would then only be to select the right cell types from a pool of already lineage-restricted cells (3). Cell-intrinsic transcription factors (4–7) and activation of ectopically expressed cytokine receptors (8–10) were shown to instruct lineage decisions. However, because of technical limitations, the instructive action of cytokines acting physiologically on unmanipulated HPCs could not be demonstrated (1, 11). As illustrated in fig. S1 (12), the discontinuous analysis of HPCs does not allow conclusive answers (13): In order to exclude the selective model, the exact kinship, lineage commitment, and cell death of all individual cells in HPC colonies must be identified (14, 15). Prior analy-

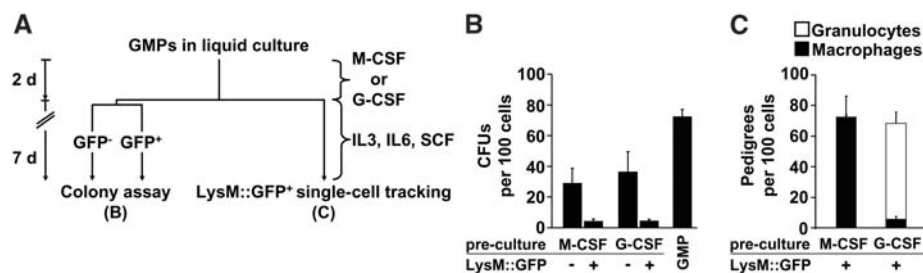
ses had not continuously followed all individual cells in constantly mixing HPC cultures long enough and with sufficient resolution. The selective model is currently favored in the literature (11, 16).

Using bioimaging approaches that allowed continuous long-term observation at the single-cell level (17) (fig. S2 and movies S1 to S4), we analyzed cultures of murine granulocyte-macrophage progenitors (GMPs) (18) (fig. S3) in the presence of only macrophage- or granulocyte colony-stimulating factor (M- or G-CSF). Single-cell tracking showed that GMPs functionally respond to both cytokines with high cloning efficiency (figs. S4 and S5). Culture in only M- or G-CSF leads almost exclusively to mature monocytic (M) or neutrophil granulocytic (G) cells,

respectively (fig. S6). We utilized LysM::GFP mice (19), expressing enhanced green fluorescent protein (GFP) from the *lysosomeM* gene locus as an early molecular reporter for unilineage commitment. Whereas only extremely weak LysozymeM::GFP expression (LysM::GFP<sup>-</sup>) is found in undifferentiated GMPs, LysM::GFP is drastically up-regulated (LysM::GFP<sup>+</sup>) upon differentiation (19–21) (figs. S2B and S7 to S9 and movie S5). LysM::GFP<sup>+</sup> cells have lost their colony-forming potential (Fig. 1, A and B, and fig. S9) and are unilineage-restricted to either the M or G lineage (Fig. 1, A and C).

This approach allows detection of cell death and unilineage commitment of all cells in GMP cultures. We continuously observed hundreds of GMPs and all of their progeny throughout development into only M- or G-committed cells in the presence of only M- or G-CSF (375 pedigrees for M-CSF, 318 for G-CSF) (figs. S10 and S11). Colonies without cell death can be explained in two ways: (i) the colony-initiating cell was a bipotent GMP, and with the absence of selective cell deaths, it must have differentiated exclusively into the lineage supported by the present cytokine, or (ii) the colony-initiating cell was already unilineage-restricted to this lineage (Fig. 2A). We determined that the original GMP population contained a maximum of 23 ± 6% and 53 ± 7% potentially unilineage-restricted M and G cells, respectively (fig. S3).

Quantifying the frequency of GMP pedigrees without cell death in single-cytokine conditions allowed us to identify the lineage-instructive effect of M- and G-CSF. In 88 ± 2% (M-CSF) and



**Fig. 1.** LysM::GFP is a marker for unilineage-restricted G or M cells. (A) Experimental procedure. (B and C) LysM::GFP<sup>+</sup> cells derived from GMPs cultured with only M- or G-CSF for 48 hours have lost colony-forming potential (B) and are unilineage-restricted (C). Means ± SD of 50 pedigrees per condition and experiment ( $n = 3$ ). CFU, colony-forming unit; IL, interleukin; SCF, stem cell factor.

Institute of Stem Cell Research, Helmholtz Zentrum München—German Research Center for Environmental Health, Neuherberg-Munich, Germany.

\*To whom correspondence should be addressed. E-mail: timm.schroeder@helmholtz-muenchen.de