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Ras Oncoproteins Transfer from Melanoma Cells to T Cells and Modulate Their Effector Functions

Helly Vernitsky,*† Oded Rechavi,*†† Nir Rainy,* Michal J. Besser,‡ Meital Nagar,*†† Jacob Schachter,‡ Yaniv Lerenthal,‡ Marcelo Ehrlich,§ Yoel Kloog,*‡** and Itamar Goldstein*†,‡

Lymphocytes establish dynamic cell–cell interactions with the cells they scan. Previous studies show that upon cell contact, various membrane-associated proteins, such as Ras-family proteins, transfer from B to T and NK lymphocytes. Mutations in RAS genes that encode constitutively active, GTP-bound, oncoproteins are rather common in human cancers; for instance, melanoma. Cancer immunoediting has been postulated to contribute to the elimination of malignant melanoma. Thus, we asked whether Ras oncoproteins can transfer from melanoma to T cells, including tumor-infiltrating lymphocytes (TILs), and subsequently induce functional effects in the adopting T cells. To explore this issue, we genetically engineered an HLA-A2+ melanoma cell line, MEL526, to express GFP or GFP-tagged H-Ras mutants stably. In this study, we show by an in vitro coculture system that GFP-tagged H-Ras, but not GFP, transfers from MEL526 to T cells and localizes to the inner aspect of their plasma membrane. This cell-contact-dependent process was increased by TCR stimulation and did not require strict Ag specificity. Importantly, we found a positive correlation between the levels of the acquired constitutively active H-RasG12V and ERK1/2 phosphorylation within the adopting TILs. We also show a significant increase in IFN-γ production and cytotoxic activity in TILs that acquired H-RasG12V compared to TILs that acquired a different H-Ras mutant. In conclusion, our findings demonstrate a hitherto unknown phenomenon of intercellular transfer of Ras oncoproteins from melanoma to TILs that consequently augments their effector functions. The Journal of Immunology, 2012, 189: 4361–4370.

Immune cells establish dynamic and highly adhesive cell–cell interactions with the cells they survey, which occurs at a specific contact region (1). This cellular interface has been termed the immunological synapse. Lymphocytes also form structural continuity with the cells they survey, including plasma membrane fusions and bridges (2), gap junctions (3), tunneling nanotubes (4), and other “pores” through which individual lymphocytes become physically integrated with the target cells they

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Abbreviations used in this article: CBA, cytometric bead array; FSC, forward side scatter; MCSP, melanoma-associated chondroitin sulfate proteoglycan; MFI, mean fluorescence intensity; PI, propidium iodide; PM, plasma membrane; PP2, 4-amino-5-(4-chlorophenyl)-7-(dibutyrylamino)-3,4-dipyridimidine; SILAC, stable-isotope labeling of amino acids in cell culture; SSC, side scatter; TIL, tumor-infiltrating lymphocyte.

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determination of cell fate as well as oncogenic transformation (12). Somatic mutations, for example, in N-Ras (or its molecular target B-RAF) are present in a large percentage of human melanoma (13). Cancer immunoediting is postulated to be involved in the elimination of malignant melanoma (14), and moreover immunotherapy by adoptive transfer of autologous melanoma-specific tumor-infiltrating lymphocytes (TILs) has been reported to increase the survival of patients with advanced melanoma with some patients even achieving complete remission (15, 16).

Thus, it was of interest to ask whether mutated Ras oncogenes transfer from cancer cells, such as melanoma, to T cells and to explore the role of the protective functions of this transfer. To address this issue, we created stable MEL526 transfectants expressing GFP-H-RasG12V, GFP-H-Ras-C184S, or GFP. In this study, we show by an in vitro experimental system of TILs or peripheral blood-derived T cells cocultured with various MEL526 transfectants that GFP-tagged H-Ras proteins, but not GFP, transfer during contact from MEL526 into T cells. Moreover, we demonstrate that the acquired active H-RasG12V oncoproteins augment various effector functions in the adopting TILs.

Materials and Methods

Human subjects

This study was approved by the Institutional Ethics Committee at the Chaim Sheba Medical Center. All peripheral blood samples and primary T cells were obtained from healthy blood donors that signed an informed consent. Melanoma patients at stages III and IV undergoing a surgical resection of metastatic melanoma lesions for clinical indications all signed an informed consent to provide tumor tissue and participate in the study.

Abs and reagents

Fluorochrome-conjugated anti-CD3, anti-CD4, and anti-CD8 mAbs were obtained from BD Biosciences. Anti–melanoma-associated chondroitin sulfate proteoglycan PE-conjugated mAbs were from Miltenyi Biotec. Purified anti–p-ERK mAbs and anti-GFP mAbs were from Sigma and anti-Ras from Calbiochem. Alexa Fluor 647-conjugated mAbs against CD107a were from BD Biosciences, and Alexa Fluor 488-conjugated secondary Abs against mouse IgG were obtained from Molecular Probes (Invitrogen).

Plasmids and transfection

The expression vectors containing cDNA encoding GFP, GFP-H-RasG12V, were previously described (8). MEL526 cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) as indicated in the product data sheet and selected to express the indicated vector stably.

Isolation of primary T cells

PBMCs were isolated by density-gradient centrifugation on Histopaque 1077 (Sigma), as previously described (17). To obtain a population enriched for lymphocytes and T cells, PBMCs were cultured for 16 h in plastic wells and, the nonadherent cells (~85% CD3+ T cells) were collected.

TILs and primary T cell cultures

After surgical resection of metastatic melanoma lesions, minimally cultured unselected, short-term cultures of “young” TILs were established, as previously described (18). The cultured TILs were then expanded to clinical-treatment levels in a rapid expansion procedure, using anti-CD3 Abs (Orthocline OKT-3; Cilag), a medium supplemented with 2000 IU/ml rhIL-2 (Proleukin; Chiron B.V.), and irradiated feeder cells, as previously described (17). In general, within 14 d, the cultured TILs expanded by multiple folds, and a portion of the cells was used for the various in vitro experiments described later. The MART-126–35–specific HLA-2–restricted CD8+ T cell clone 2e2 generated from HLA-2+ TILs of a patient with metastatic melanoma (20) was kindly provided by Michal Lotem (Hadassah-Hebrew University Hospital, Jerusalem, Israel).

The CD3+ T cell-enriched PBMCs were activated by plate-bound OKT3 mAbs (2 μg/ml) and soluble anti-CD28 mAbs (1 μg/ml; BD Biosciences) in complete RPMI 1640 medium (containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, all from Life Technologies) and supplemented with 100 IU/ml rhIL-2. Seventy-two hours later, at a time point when most of the nonadherent cells were CD3+ T cells (>90%), the cultures were harvested, and cells were further used as indicated.

Melanoma cell lines

The human cell lines HLA-2+ MEL526 and HLA-2– MEL938 were cultured in RPMI 1640 supplemented with 10% FBS and maintained at 37°C in a humidified 5% CO2 incubator.

Cocultures and FACS analysis of intercellular transfer

To examine the intercellular transfer of GFP-tagged molecules, the various MEL526 transfectants were distributed into FACS tubes (3 × 106 cells/tube in 100 μl) to which were added TILs or primary T cells (6 × 105 cells/ml in 100 μl) to obtain an E:T ratio of 2:1. The tubes were centrifuged for 2 min at 1000 rpm to promote cell-conjugate formation and then cocultured for the specified time at 37°C. In some experiments, the T cells were pretreated for 30 min with one of the compounds latrunculin A/B (10 μM), cytochalasin B (100 μM), and 1,4-diamino-2-[(4-chlorophenyl)azo]pyrazolo[3,4-d]pyrimidine (PP2; 100 μM), or EDTA (5 mM) and then cocultured with MEL cells for an additional 5 h either in the presence of the compound or in control medium (1% DMSO).

Microscopy

For spinning disk confocal imaging (Zeiss 200M microscope, CSU-22 Yokogawa spinning disk, ×100 1.4 NA lens, Photometrix H2Q camera; all under the control of Slidebook software), cells were fixed in 2% paraformaldehyde, cytopsin onto slides, and the T cells were identified by immunostaining with anti-CD45 PE–Cy5 mAbs. Z-stacks were acquired at 0.3-μm steps. Image deconvolution was done by the Nearest Neighbors algorithm of Slidebook, which was also used for the three-dimensional rendering.

Transwell assay

TILs and the various T cells were prevented from directly contacting the MEL526 by a Transwell 0.4-μm-pore membrane (Costar), as described previously (8, 21). Briefly, 5 × 106 effector cells (in 0.3 ml of medium) were either added to MEL526 transfectants (0.5 ml of medium) in the lower compartment or placed in the upper chamber separated from targets (assay performed in 24-well plates). The cultures were incubated for 3 h, and the cells from the lower chamber were collected and analyzed for GFP-H-RasG12V acquisition by T cells.

FACS analysis and cell sorting

Prior to the FACS-based analysis, the cells were stained with anti-CD3 allophycocyanin mAbs to identify T cells. After immunostaining to obtain a single-cell suspension, the cell pellets were resuspended vigorously in ice-cold 5 mM EDTA/PBS, vortexed, passed through a 35-μm cell strainer (BD Biosciences), and kept on ice until the analysis. To include in the final analysis only viable TIL singlet cells, we used a stringent multiparametric gating strategy, as described elsewhere (8, 10, 22). Briefly, viable T cells were identified by their expression of CD3 and distinct forward side scatter (FSC) and side scatter (SSC), including pulse width, height, and area (see Fig. 1Ai–iii). In preliminary experiments, by costaining with propidium iodide (PI), we further verified that our gating algorithm to identify viable singlet T cells indeed excluded PI+ dead cells (Supplemental Fig. 1A). We found that gating out PI+ events in addition to our standard gating procedure to identify viable singlet T cells did not modify the results appreciably (Supplemental Fig. 1A).

Samples were acquired on a FACS Calibur using CellQuest software or on a FACS Aria instrument using FACSDiva software (all from BD Biosciences). All cell-sorting experiments were performed on a FACS Aria instrument. Collected data per sample included >10,000 singlet T cell events, which were analyzed using the FlowJo 7.2.5 software (Tree Star).

Intracellular staining for phosphoflow analysis

Intracellular phosphoprotein staining for flow cytometry was performed as previously described (17, 23). Briefly, cells were fixed in 2% paraformaldehyde followed by permeabilization with ice-cold methanol. Next, the cells were stained with a primary anti-phospho–specific ERK1/2 MAb for 20 min at room temperature, washed, and stained with a secondary goat anti-mouse–Alexa Fluor 647 Ab.

CD107a degranulation assay

CD107a+ lysosome-associated membrane glycoprotein (LAMP1) present in CD107a degranulation assay was performed as previously described (24). The FACS-based CD107a+ degranulation assay was performed as previously described (24).
Briefly, TILs were cocultured alone or with MEL526 transfectants for 6 h. At coculture initiation, anti-CD107a–Alexa 647–conjugated mAbs were added (eBioscience) together with 10 μg/ml brefeldin A (Sigma). Subsequently, harvested cells were analyzed for CD107a degranulation by FACS analysis, as described earlier.

ELISA

p-ERK1/2 levels were determined in the indicated cell lysates by an ELISA kit (ERK1/2, Phospho ELISA Development Kit; R&D Systems). The various cells were lysed and samples analyzed in duplicate according to the manufacturer’s instructions. All reagents needed were supplied within the kit.

Cytokine detection

For intracellular cytokine detection, TILs were cultured either alone or with MEL526 transfectants for 5 h in the presence of brefeldin A (Golgi Plug). Then, the cells were harvested, fixed, and permeabilized using the Cytofix/ Cytoperm Plus kit and stained with anti–IFN-γ mAbs (all reagents from BD Biosciences).

Alternatively, TILs were placed in U-bottom 96-well plates (1 × 10^5 cells/well in 100 μl of medium) to which were added MEL526 transfectants (5 × 10^4 cells/well in 100 μl of medium). Supernatants were collected 24 h later, and levels of IFN-γ were assayed, as previously described (8), using a cytometric bead array (CBA) assay (from BD Biosciences). Data were collected on a FACS Calibur and analyzed using the BD Biosciences CBA software according to the manufacturer’s instructions.

Statistical analysis

The p values were calculated by Student t test, and p < 0.05 was considered significant.

Results

GFP-H-RasG12V transfers from MEL526 transfectants to T cells during coculturing

First, we wanted to ask whether H-RasG12V can transfer from tumor cells into lymphocytes. To address this question in the context of malignant melanoma, we generated HLA-A2+ MEL526 cells stably expressing the GFP-tagged oncoprotein H-RasG12V (termed in this study MEL526-GFP-H-RasG12V) or GFP alone (MEL526-GFP). In parallel, we expanded various short-term lines of unselected “young” HLA-A2+ and HLA-A2–CD3+ TILs, as described in Materials and Methods. Of note, most of these TIL lines were composed of a mixture of CD4+ and CD8+ CD3+ T cells. Subsequently, we cocultured the different TILs with the MEL526 trans-
fectants at a 2:1 ratio. At the end of coculturing, the cells were immunostained with anti-CD3 mAbs, and prior to FACS analysis we used various techniques to disrupt cell conjugates (see Materials and Methods for details). To distinguish in the final multiparametric digital flow cytometry data analysis with high probability T cell singlet events from T cell/MEL526 conjugates, dead cells, and cellular debris, we used very stringent gating criteria based on CD3" staining and the typical light-scattering characteristics of viable singlet T cells that compose a population of homogenously spherical cells (Fig. 1A-i–iii). First, using this coculture system and analysis algorithm, we found that after 3 h of coculturing of HLA-2" TILs with MEL526-GFP-H-RasG12V or MEL526-GFP, a large percentage of the CD3" singlet T cells acquired H-RasG12V but not GFP (Fig. 1A, histogram overlay iv).

To rule out that TIL/MEL526 conjugates were included in the analyses for H-Ras transfer, we further verified, prior to using our standard gating algorithm, that CD3" events that stained positive for melanoma-associated chondroitin sulfate proteoglycan (MCSP; a transmembrane melanoma-specific Ag) were all excluded from the final T singlet cells gate. As shown in Fig. 1B, we formed a gate specific for CD3"MCSP" events in the TIL/MEL526 cocultures that was first set based on similar TILs cultured alone (Fig. 1B, plots ii and i, respectively). The results indeed confirmed that GFP-H-RasG12V— but not GFP—is acquired by a large percentage (64.93%) of the singlet CD3"MCSP" (Fig. 1B, histograms iv and iii, respectively).

Next, we compared the mean fluorescence intensity (MFI) of the GFP-H-RasG12V signal in CD8"HLA-2" versus HLA-2" TILs after coculturing with MEL526-GFP-H-RasG12V for 3 h (Fig. 1A, left histogram overlay). By overlaying the FACS data histograms, we found that usually CD8"HLA-2" singlet TILs acquired significantly more GFP-H-RasG12V than HLA-2" TILs (MFI = 2132 versus 900, respectively, p < 0.01). Of note, the percentage of CD4" and CD8" TILs that acquired GFP-H-RasG12V during coculturing was typically comparable (Supplemental Fig. 1B, p = NS; n = 5).

To address further how important was strict TCR-specificity for H-RasG12V transfer, we next cocultured polyclonal T cells derived from PBMCs of healthy donors with MEL526-GFP-H-RasG12V or control MEL526-GFP for 3 h. We found that unselected polyclonal CD3" singlet T cells could acquire GFP-H-RasG12V, but not GFP, during coculturing (Fig. 1A, right histogram overlay). However, prior in vitro stimulation with OKT3 mAbs further increased this capacity (MFI = 803 versus MFI = 483, p < 0.01).

We also found that the transfer of GFP-H-RasG12V to TILs was substantial by 30 min and moreover the degree of transfer increased very minimally over the next 24 h of coculturing (Supplemental Fig. 1C).

To confirm conclusively that GFP-H-RasG12V transfers to singlet TILs, we physically sorted out from MEL526-GFP-H-RasG12V/TILs cocultures two populations of singlet CD3" TILs based on their GFP content. The cutoff among GFP" and GFP" events was determined by a parallel analysis of control pure TILs (left upper plot). Numbers in parentheses represent the MFI of the GFP signal in the specified CD3" singlet TILs population. Data are from a typical experiment of >3 performed.

The percentage of the CD3" singlet T cells acquired H-RasG12V but not GFP (Fig. 1A, histogram overlay iv). The results indeed confirmed that GFP-H-RasG12V— but not GFP—is acquired by a large percentage (64.93%) of the singlet CD3"MCSP" (Fig. 1B, histograms iv and iii, respectively).

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Next, by time-lapse confocal videomicroscopy, we could image the progression of GFP-H-RasG12V acquisition from MEL526. In Supplemental Video 1, we show a red-labeled MEL526-specific CD8"HLA-2"2ε2 TIL forming tight contact with a green MEL526-GFP-H-RasG12V cell and instantly starting to acquire large quantities of GFP-H-RasG12V. Subsequently, the CD8"cytotoxic TIL also induces the lysis of the conjugated MEL526 cell; all occurring within ~20 min.

Thus, RasG12V transfers from MEL526 transfectants to bona fide CD3" singlet T cells in a process that is enhanced by prior TCR activation and that does not require strict TCR specificity.

Acquired H-RasG12V is intracellular and localizes to the adopting T cell's plasma membrane

Previous studies imply that cell surface receptors transferred among lymphocytes assume a normal in/out transmembrane orientation in the adopting lymphocyte (6, 25), an observation found to be valid also for the transfer of Ras among 721.221 B cells and NK cells (8). We now asked whether the acquired GFP-H-RasG12V molecules are primarily incorporated into the intracellular plasma membrane (PM) of the adopting TILs, and thus, we used immunofluorescence staining with anti-Ras or anti-GFP mAbs to determine whether the transferred GFP-tagged Ras is inaccessible to the Abs (i.e., intracellular) or alternatively can be detected on the PM. By FACS analysis, we found a positive correlation between the MFI of anti-Ras or anti-GFP staining and the amount of GFP-H-RasG12V acquired by the TILs only in permeabilized TILs. In contrast, without permeabilization, no significant anti-Ras or anti-GFP staining was detected (Fig. 3A, 3B; red lines depict trend lines drawn by hand).

Next, by confocal microscopy, we show in a TIL/MEL526-GFP-H-RasG12V conjugate that formed during coculture that the green GFP-H-RasG12V molecules that ignored cell boundaries are mostly distributed in the PM of the adopting TIL, similarly to their predominant PM distribution in the donor MEL526 cell (Fig. 3C).

Together, all these multimodality data suggest that the transferred GFP-H-RasG12V was in fact incorporated into the inner aspect of the PM of the adopting TILs.

Acquired H-RasG12V induces increased ERK1/2 phosphorylation in the adopting T cells

The main signal cascade induced by activated GFP-bound Ras in T cells is composed of MAPK (ERK1/2), MAPKK (MEK1/2), and MAPK (ERK1/2) (26, 27). To answer whether H-RasG12V can indeed signal in the adopting TILs, we first analyzed by flow cytometry the correlation among GFP-H-RasG12V acquisition and intracel-
lular p-ERK1/2 levels in adopting T cells (Fig. 4A). We found that p-ERK levels within TILs that acquired GFP-H-RasG12V from MEL526-GFP-H-RasG12V were significantly higher than in their GFP−/dim counterparts (MFI = 243 versus MFI = 134, respectively, p < 0.001) or when compared with similar TILs cocultured with control MEL526-GFP (MFI = 243 versus MFI = 119, respectively, p < 0.001). In addition, using the FlowJo software, we exported the scaled raw data as a CSV file for additional statistical analysis. This analysis, depicted as scatterplots with best-fitting linear trend line (Fig. 4B; plots generated using Microsoft Excel), further emphasized that there was a statistically significant positive correlation between GFP-H-RasG12V acquisition and p-ERK1/2 levels in the adopting T cells [Fig. 4Biv; Spearman's coefficient of rank correlation (\(r\)) = 0.445, p < 0.001; calculated using MedCalc v11, MedCalc Software bvba, Mariakerke, Belgium]. Importantly, a rather similar level of positive correlation (\(r\) = 0.532; p < 0.001) among GFP-H-RasG12V and p-ERK1/2 levels was also found within the RasG12V-donor MEL526 transfectants (Fig. 4Bii). In contrast, we did not find such a positive correlation in control MEL526 cells (Fig. 4Bi) or control T cells cocultured with the latter cells (Fig. 4Bii).

In similar experiments, we cocultured TILs from different patients with MEL526-GFP-H-RasG12V and sorted out GFP-H-RasG12V+ and GFP-H-RasG12V−/dim TILs. As an additional control, we also sorted out TILs from TIL/MEL526-GFP cocultures. Then, the various cell lysates were analyzed for p-ERK1/2 levels by prestandardized ELISA kits (from R&D Systems). As an additional "quality control," we also compared among p-ERK levels in the lysates of MEL526-GFP, MEL526-GFP-H-RasG12V, and parental MEL526. The analysis of the pooled data from \(n\) = 3 different experiments (Fig. 4B) showed that p-ERK1/2 levels were significantly increased in the sorted-out singlet H-RasG12V+ TILs compared with their RasG12V−/dim counterparts (p < 0.05) or compared with control TILs (p < 0.01).

Acquisition of H-RasG12V by TILs is linked to increased IFN-\(\gamma\) production

Activation of the Ras/ERK1/2 cascade in vitro has been linked to increased IFN-\(\gamma\) secretion from NK cells (28) and TILs isolated from human renal cell cancers (29). Thus, it was of interest to assess the functional consequences of the acquisition of GFP-H-RasG12V on the adopting TILs by specifically analyzing the production of IFN-\(\gamma\) in GFP-H-RasG12V+ compared with GFP-H-RasG12V− effector TILs. To address this question, we used a system whereby various MEL transfectants were cocultured with MEL526-specific TILs and treated with a Golgi transport inhibitor to promote intracellular accumulation of the cytokines. The single-cell FACs analysis showed that GFP-H-RasG12Vhi TILs produced significantly more IFN-\(\gamma\) compared with their GFP-H-RasG12V−/dim counterparts or similar TILs cocultured with control MEL526-GFP (MFI = 1098 versus MFI = 561 or 908, respectively; p < 0.01 for both differences; Fig 5A, see related gray-shaded regions).
addition, we stably transected MEL526 with a GFP-tagged H-Ras mutant, C184S, which is only monopalmitoylated and hence demonstrates aberrant function (30). We found that although GFP-H-Ras-C184S was acquired by the majority of TILs during co-culture, their overall IFN-γ production was significantly reduced compared with similar TILs that acquired the constitutively active GFP-H-RasG12V oncoprotein (MFI = 1098 versus MFI = 342, respectively; \( p, 0.001 \); Fig 5A, related gray-shaded regions).

Next, we analyzed IFN-γ levels in the supernatants of various TILs \( (n = 3) \) cocultured with either MEL526-GFP or MEL526-GFP-H-RasG12V cells for 24 h by a FACS-based CBA assay (Fig. 5B). We found that IFN-γ secretion was significantly higher in the TILs cocultured with MEL526-GFP-H-RasG12V compared with TILs cocultured with control MEL526-GFP or alone (\( p < 0.05 \) and \( p < 0.01 \), respectively).

Thus, the data imply a link among the levels of “non–cell-autonomous” GFP-H-RasG12V acquired by TILs and their capacity to produce IFN-γ.

**Acquisition of H-RasG12V is linked to augmented CD107a degranulation**

Because activation of ERK1/2 has been suggested to increase the cytolytic activity of CTLs (31) and TILs (29), we next asked whether the acquisition of GTP-bound RasG12V can enhance this function. To test this issue, we initially used a MART-1 26–35–specific HLA-2–restricted CD8 + T cell clone, 2e2, generated from TILs of a patient with metastatic melanoma (see Materials and Methods for details). First, we determined that the 2e2 TILs indeed displayed specific cytotoxicity toward HLA-2 + MEL526 but not toward HLA-A2 MEL938 cells by the FACS-based CD107a degranulation assay (Fig. 6A, lower plots). As quality controls, we also analyzed TILs cultured with medium alone or activated by anti-CD3/CD28–coated Dynabeads (Fig. 6A, upper plots). Next, to address directly whether H-RasG12V acquisition was associated with increased CD107a degranulation, we compared among 2e2 cocultured with MEL526 engineered to express stably GFP-H-RasG12V, GFP-H-RasC184S, or GFP (Fig. 6B). We found that the MFI of CD107a degranulation was significantly higher in H-RasG12V+ compared with their counterpart RasG12V−/dim singlet 2e2 T cells after coculturing with MEL526-GFP-H-RasG12V (MFI = 208 versus MFI = 120, respectively, \( p, 0.01 \)). Importantly, 2e2 T cells that acquired the GFP-H-RasC184S mutant showed significantly reduced CD107 degranulation compared with 2e2 T cells that acquired H-RasG12V (MFI = 125 versus MFI = 208, respectively, \( p < 0.01 \)).
Numbers in parentheses represent MFI of the IFN-γ were submitted to fixation and permeabilization and immunostained or alone for 5 h in the presence of brefeldin A. After the incubation, cells MEL526-GFP, MEL526-GFP-H-RasG12V, MEL526-GFP-H-Ras-C184S, FACS-based CBA assay, as detailed in Materials and Methods contained in the relevant gray-shaded region. Data from ∼6

After 24 h, supernatants were collected and IFN-γ production by TILs. (∼6)

FIGURE 5. Acquisition of GFP-H-RasG12V is associated with increased IFN-γ production by TILs. (A) HLA-2+ TILs were cocultured with MEL526-GFP. MEL526-GFP-H-RasG12V, MEL526-GFP-H-Ras-C184S, or alone for 5 h in the presence of brefeldin A. After the incubation, cells were submitted to fixation and permeabilization and immunostained with fluorochrome-conjugated anti–IFN-γ mAbs and analyzed by FACS. Numbers in parentheses represent MFI of the IFN-γ signal in singlet TILs contained in the relevant gray-shaded region. Data from ∼10,000 singlet T cells were collected on a FACSAria instrument and analyzed using FlowJo V7.2.5 (shown is a typical experiment of n = 3 performed). (B) TILs were cocultured with either MEL526-GFP, MEL526-GFP-H-RasG12V, or alone. After 24 h, supernatants were collected and IFN-γ measured by a FACS-based CBA assay, as detailed in Materials and Methods. Bars represent mean ± SD of IFN-γ (pg/ml) secreted by the different TILs, as indicated in the key (data were pooled from n = 3 experiments).

Moreover, we repeated the CD107a degranulation assay with a variety of unselected short-term cultured TILs (n = 7) and found that CD107a degranulation (determined by ΔMFI relative to control TILs cultured alone) was significantly increased in H-RasG12V+ versus RasG12V−dim singlet TILs (Fig. 6C, p < 0.05).

Thus, GFP-H-RasG12V acquisition from MEL526 during coculturing and the resulting increase in p-ERK1/2 levels is also associated with increased cytotoxic capacity.

Analysis of cellular processes involved in the transfer of H-RasG12V from MEL526 to TILs

In previous studies (8, 10), we showed that Ras transfer from 721.221 to NK cells—where a typical immune synapse forms among the two coculture partners (32)—was contact-dependent and very sensitive to inhibition by low concentrations (0.5–1 μM) of latrunculin B, which prevents polymerization of actin monomers. Hence, we tested whether Ras transfer from MEL526 to TILs was, likewise, highly dependent on a functional actin cytoskeleton. Distinctly from our previous observations, RasG12V transfer was only partly inhibited by latrunculin A or latrunculin B, even if in a statistically significant manner (p < 0.05). Moreover, this partial inhibition was produced only under serum-free conditions and required high concentrations of ≥10 μM (Fig. 7A, right bar graph). Next, we tested the effect of additional compounds that interfere with the normal function of the actin cytoskeleton: cytochalasin B and the Src family kinase inhibitor PP2 (6, 25). We found that both produced a moderate, yet statistically significant (p < 0.05), inhibition of RasG12V transfer.

Consistent with previous studies (5–10), we confirmed that Ras transfer was unequivocally cell contact dependent; for instance, when the TILs were separated from the MEL526-GFP-H-RasG12V by a semipermeable Transwell membrane (0.4-μm pore size), the transfer was completely inhibited (Fig. 7A, left bar graph). Additionally, EDTA, which interferes with cation-dependent cell–cell adhesion, strongly decreased the transfer by ∼80% (Fig. 7A, left bar graph; p < 0.05).

Our data also show that MEL526 lysis was not essential for the transfer of GFP-H-RasG12V to TILs. As shown in Fig. 7B, when we tested TILs from different patients (n = 10), we did not detect correlation between their cytotoxicity (i.e., CD107a degranulation) and their capacity to acquire GFP-H-RasG12V during coculturing (Pearson correlation coefficient R² = 0.02).

Together, these results suggested that the transfer of RasG12V from melanoma to TILs is highly contact dependent and partially dependent on a fully functional actin cytoskeleton, whereas target cell killing is not a requirement.

Discussion

This study reveals a hitherto unknown process whereby T cells acquire during cell contact from cancerous melanoma cells the constitutively active, membrane-associated, oncoprotein H-RasG12V. Our data, moreover, show that this transfer has the potential to serve as a “danger signal” that enhances IFN-γ production and cytotoxic activity within TILs that acquired H-RasG12V.

In this work, as a proof of concept, we only focused on a single Ras oncogene, but our current results do not place a limit on the types and numbers of signaling proteins that can transfer among cancer and immune cells. In this regard, we had conceived a novel quantitative proteomics platform that identifies with high fidelity cancer and immune cells. In this regard, we had conceived a novel quantitative proteomics platform that identifies with high fidelity non–cell-autonomous proteins acquired by lymphocytes during cell–cell contact. By this method, we have recently determined that a multitude of proteins (∼200) transfer among 721.221 B cells and primary human NK cells (10). This list was primarily composed of membrane-associated proteins, including endogenously expressed Ras and other small GTPases of the Ras superfamily (e.g., Rab, Ral, and Arf). It is thus reasonable to assume that, likewise, lymphocytes acquire many proteins in vivo from cancer cells during immunosurveillance.

In the past two decades, major advances have been made in the understanding of numerous aspects of anti-cancer immunity (33, 34). These studies led to further refinement of the classical immunosurveillance theory by Schreiber and Smyth, putting forward the concept of cancer immunoediting that consists of three phases: elimination, equilibrium, and escape (34). However, various aspects of the transfer of information from cancer to immune cells and how this transfer regulates immunoediting of cancers are hitherto unknown.

The classical model of cellular immunity is based on transfer of intercellular communication through interactions between cell-surface proteins.
receptors and their respective ligands (1, 35). The current finding that T cells acquire intact active H-RasG12V from melanoma cells during contact suggests a new mechanism, whereby T cells normally probe selected parts of the target cell’s proteome and can consequently respond to the transfer of mutated oncoproteins. One limitation is that in situ, the TILs to tumor cells ratio is typically

FIGURE 6. GFP-H-RasG12V acquisition during coculturing is linked to increased CD107a degranulation. (A and B) MART-126–35–specific 2e2 TILs were cocultured for 6 h with various HLA-A2+ MEL526 transfectants or HLA-A2+ MEL938 cells or alone. Stimulation with anti-CD3/CD28-coated Dynabeads served as a positive control. To determine the cytolytic activity of 2e2 TILs, we added anti-CD107a mAbs and brefeldin A (Golgi transport inhibitor) at coculture initiation. The various pseudocolor density plots show CD107a-degranulation level detected in CD3+ singlet TILs versus SSC (A) or GFP signal (B). Numbers in parentheses (B) represent the MFI of CD107a signal in the singlet TILs contained in the relevant gray-shaded region. Data are from a typical experiment of >5 performed (data collected from ~10,000 singlet T cells). (C) The bar graph shows the pooled data (mean ± SD; logarithmic scale) from a variety of unselected short-term TILs from different patients cocultured with MEL526-GFP-H-RasG12V. The ΔCD107a MFI was calculated relative to control TILs cocultured with MEL526-GFP (data were pooled from n = 7 experiments). *p < 0.05.

FIGURE 7. RasG12V transfer is cell contact dependent and partly regulated by the actin cytoskeleton but target-cell-killing independent. (A) The coculture partners were preincubated for 1 h with the various indicated compounds. Subsequently, TILs were cocultured with MEL526-GFP-H-RasG12V cells for 3 h in the presence of the indicated compound or the indicated treatment. Acquisition of GFP-H-RasG12V by TILs under the different experimental conditions was then determined by FACS analysis (data collected from ~10,000 single T cell events). Experiments were performed in a complete RPMI 1640 medium with 10% FCS (left panel) or without serum (right panel). Percent GFP-H-RasG12V transfer was determined relative to the medium without treatment cocultures (set as 100% transfer). Data are from a typical experiment of >5 performed. *p < 0.05. (B) The scatterplot depicts cytolytic activity (% CD107a degranulation; x-axis) against the percentage of TILs that acquired GFP-H-RasG12V (y-axis) during coculturing with MEL526-GFP-H-RasG12V (data obtained from 10 different TILs). The linear trend line and the Pearson correlation coefficient (R²) were calculated using Microsoft Excel software.
very low compared with our in vitro system, and moreover data regarding the in vivo significance of this phenomenon are not yet available.

Notably, we found that the acquisition of H-RasG12V by T cells from HLA-2+ MELS26 was not strictly Ag specific, as both HLA-2+ and HLA-2− TILs as well as polyclonal unselected blood-derived T cells displayed such a capacity; although it was improved by Ag specificity and TCR stimulation. In contrast, the process of trogocytosis (“membrane snatching”) has been postulated to be more closely associated with specific TCR recognition (5, 20). In this regard, Hudrisier and colleagues (36) analyzed the transfer of a large panel of proteins with different types of anchors to the PM from APCs to lymphocytes. They found that Ras family proteins (H-Ras and K-Ras) were among the most efficiently transferred, and distinctively their transfer from HEK transfectants to TILs was improved by Ag specificity and TCR stimulation. In contrast, the transfer of a large panel of proteins with different types of anchors to the PM from APCs to lymphocytes was among the most efficiently transferred, and distinctively their transfer from HEK transfectants to TILs was increased by promoting a specific interaction, yet the transfer was increased by promoting a specific interaction.

In T cells, a set of adaptors link TCR signaling to the two guanine nucleotide exchange factors RasGRP1 and Sos1 (26), which catalyze the activation of Ras, and consequently its main signal cascade in the adapting T cells composed of MAPKKK (Raf), MAPK (MEK1/2), and MAPK (ERK1/2). There is even evidence that the threshold of TCR activation during its interactions with peptide-MHC complexes is partly set by the different strength of activation of the Ras/MAPK cascade (37). Activated p-ERK1/2 dimers can regulate many targets in the cytosol and also translocate to the nucleus where they activate a variety of transcription factors (e.g., Elk1, Ets, STAT1/3, c-Fos, p90-RSKs, etc.) that are critical for T cell activation, cytokine production, and proliferation (27, 38).

Importantly, in our unique experimental system, we indeed show that after its intercellular transfer, the non–cell-autonomous constitutively active H-RasG12V activates its major signaling cascade in the adopting T cells. We demonstrate a significant positive correlation between the intensity of H-RasG12V acquisition and ERK1/2 phosphorylation (Fig. 4). Moreover, we demonstrate a link between the acquisition of H-RasG12V and enhanced IFN-γ production and cytotoxic activity in the adopting TILs. These data are a strong proof of concept that RasG12V indeed signals “normally” in the adopting TILs. Many studies support the notion that excessive Ras signaling in T cells can promote various effector functions and even reverse T cell anergy (39, 40). For example, Prinz et al. (29) demonstrate in a very recently published study that the anergy displayed by freshly isolated TILs from human renal cell carcinoma is mediated by high levels of diacylglycerol kinase-α. They suggest that this kinase disables the Ras/MAPK cascade by catabolizing the second messenger diacylglycerol-α, which controls the activation of the guanine nucleotide exchange factor, RasGRP1. The authors also show that inhibition of diacylglycerol kinase-α augments the following Ras-signaling-dependent events: i) ERK phosphorylation; ii) cytotoxic activity; and iii) the production of IFN-γ by TILs.

Our in vitro findings should be further substantiated in a relevant animal model aimed to elucidate, in vivo, the role of this “unconventional” information transfer among tumors and T cells in the early phases of cancer immunoeediting. Obviously, such studies will require exceptionally sensitive intravital imaging technologies—not yet widely available—that can accurately detect the transfer of fluorescently tagged oncoprotein from cancer cells to TILs within nascent tumors.

In conclusion, our data imply that direct contact-dependent transfer of membrane-associated Ras oncoproteins from cancer cells to TILs can serve as a stimulatory “danger” signal that augments their anti-tumor effector functions.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1
Supplemental Figure 1. Various populations of T cells were co-cultured for 3 hrs either with MEL526-GFP-H-RasG12V or with MEL526-GFP. Thereafter, collected cells were stained with appropriate fluorochrome-conjugated mAbs and treated to disrupt cell conjugates, as detailed in materials and methods section. (A) Overlay histograms show GFP-RasG12V acquisition from MEL526-GFP-H-RasG12V by recently activated peripheral blood-derived polyclonal CD3+ T cells (green histogram), as compared to similar T cells co-cultured with MEL526-GFP (red histogram). Left overlay histograms show the analysis with propidium iodide (PI) exclusion (i.e. gating out PI+ events) and right overlay histograms was created by our standard gating algorithm without (w/o) PI exclusion. (B) Bars graph depicts the percentage (mean±SD) of CD4+ or CD8+ T cells that acquired GFP-H-RasG12V during co-culturing with MEL526-GFP-H-RasG12V. (C) The bars show the MFI of GFP-H-RasG12V acquired by short-term HLA-2- (#1) and HLA-2+ (#2) TILs from MEL526-GFP-H-RasG12V cells (logarithmic scale), at the various time points specified in the graph. Data were collected on a FACSARia instrument and analyzed using Flow Jo V7.2.5.
1. Video S1. TILs were immunostained with anti-CD3 APC-conjugated mAbs (red) and plated together with MEL526-GFP-H-RasG12V (green) onto a glass bottom 35mm petri dish. Images were obtained every 20 seconds on a multiphoton laser-scanning microscope (Zeiss LSM 7 MP imaging system).